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FACTORS AFFECTING PATHOGENICITY OF PINK ROOT FUNGUS OF ONIONS [1]

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INTRODUCTION

In a previous paper (*2*) [3] a study of a number of isolates of the causal fungus of pink root of onion (*Allium cepa* L.) was reported. It was shown that several strains produced pycnidia in culture and that others were completely sterile. Mutants that were sterile appeared regularly in old cultures. All fertile isolates produced pycnidia on roots or cotyledons of onions grown in inoculated quartz sand supplied with a balanced nutrient solution, but only one isolate, which had been single-spored twice in succession, produced pycnidia on the roots of plants grown in inoculated soil. The pycnidia of these fertile isolates were always found to bear setae, and, on the basis of this hitherto unreported fact, the fungus was transferred from *Phoma terrestris* Hansen to *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker, and Larson.

The importance of the development of varieties of onion resistant to pink root for use in areas where the disease is a limiting factor has been emphasized by Porter and Jones (*5*). A method was needed whereby large numbers of seedlings could be tested rapidly under reproducible, controlled conditions and various isolates of *Pyrenochaeta terrestris* could be used as test pathogens. In the development of such a method studies were carried out on the physiology of the fungus, on its variability in pathogenicity, and on the relation of environal factors to the development of the disease in young seedlings. When optimum conditions had been determined, the relative resistance of a series of standard varieties was found by the inoculation of seedlings. The results of the investigations on physiology, pathogenicity, and host resistance are presented in this paper.

[3] Italic numbers in parentheses refer to Literature Cited, p. 18.

METHODS AND MATERIALS

The isolates of *Pyrenochaeta terrestris*, their sporulating capacity, and their sources are listed in table 1. As a rule the strains or varieties of onions to be tested were grown from seed, but in a few cases sets were used.

TABLE 1.—*Key numbers, sources, and sporulating capacity of isolates of Pyrenochaeta terrestris used*

Key No. [1]	Source	Type of original material	Sporulation of isolates on culture medium	Collector
Cal-B-H	Berkeley, Calif	Culture	−	H. N. Hansen.
Cal-D-H	Davis, Calif	Soil	−	G. N. Davis
Cal-D-T	do	Roots	+	L. D. Leach.
Col-RF-T	Rocky Ford, Colo.	Soil	−	A. M. Binkley.
Col-FC-T	Fort Collins, Colo.	do	−	Do.
Col-FC1-T	do	do	+	Do.
Col-FC2-T	do	do	−	Do.
Col-FC-H	do	do	−	Do.
La-S	Baton Rouge, La	Roots	+	F. J. LeBeau.
La-S-S	do	do	++	Do.
La-M-T	do	do	−	Do.
Tex-H	Winter Haven, Tex	do	−	S. S. Ivanoff.
Tex-T	do	do	+	B. A. Perry.
Tex-1-S	do	do	−	Do.
Tex-2-S	do	do	−	Do.
Tex-1-H	do	do	−	Do.
Tex-1-T	do	do	−	Do.
Iowa-H	Clear Lake, Iowa	Soil	−	E. S. Haber
Iowa-i-T	Fertile, Iowa	Roots	−	W. J. Hooker.
Iowa-S	do	do	+	Do.
Iowa-T	do	do	+	Do.
Utah-O-H	Utah	Soil	−	
Utah-H	do	do	−	
Utah-T	do	do	−	
Ill-T	Burlington, Ill	Roots	+	A. M. Gorenz.
Mich-H	East Lansing, Mich	Culture	−	A. L. Andersen.
Mass-T	Amherst, Mass	Roots	−	H. M. Yegian.
Wis-M-T	Madison, Wis	do	−	A. M. Gorenz.
Wis-R-T	Racine, Wis	do	−	Do.

[1] H indicates a hyphal-tip transfer; S, a single-spore transfer; T, a mass transfer.

Clean, fine, white quartz sand was used as the host substrate in most instances. In some experiments it was contained in galvanized-iron pans, 3.5 inches deep, 12 inches wide, and 22 inches long, placed in Wisconsin soil-temperature tanks (fig. 1) in a greenhouse in which the temperature ranged for the most part from 20° to 24° C. In other experiments sand cultures were run in 6-inch crocks fitted with siphons as described by Schroeder and Walker (7). In the study of the relation of hydrogen-ion concentration of the nutrient to disease development, a sand culture with a continuous drip of nutrient, as described by Pryor (6), was used.

To prepare inoculum, transfers were made from stock cultures of *Pyrenochaeta terrestris* to petri dishes containing Czapek's dextrose-

malt agar [4] and kept at 28° C. for 6 days. Nine 6-mm. disks from the border of the fungus mats in the petri dishes were transferred to 300 cc. of Czapek's modified liquid medium [5] in 32-ounce bottles, which were incubated in a horizontal position for 10 days at 28° with daily shaking to insure adequate aeration. The fungus and medium were macerated in a Waring Blendor for 3 minutes, and 100 cc. of this inoculum (except when a different amount is indicated) diluted with 900 cc. of distilled water was mixed with 10 kg. of sand.

When pans were used, the inoculated sand formed a layer approximately 2.5 inches deep, in which were marked 8 furrows one-half inch deep. Each furrow was divided into 4 equal sections, or 32 sections per pan. Two pans were inoculated with each isolate. Each half pan was considered a replicate and 16 varieties were planted
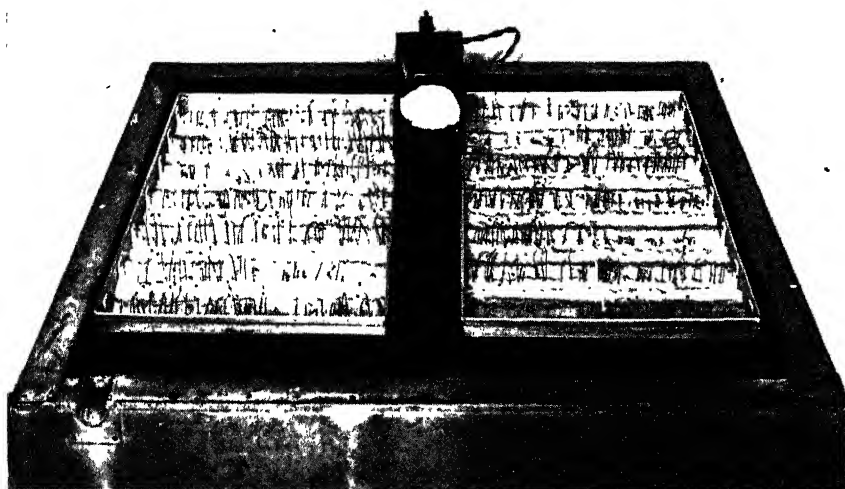
FIGURE 1.—Individual Wisconsin soil-temperature tank with culture pans.

at random in 4 replicates, so that the reliability of the data could be checked by statistical analysis. Approximately 30 seeds were planted in each section. After the seeds were planted, the pans were placed at random in the soil-temperature tanks and were covered with cardboard for 7 or 8 days; at that time the seedlings were about 1 inch high. The pans were then flooded with distilled water, the excess was siphoned off, and the pans were rewatered with the modified Hoagland's solution [6] used by Walker and Hooker (9) and designated by them as 1H. Subsequently, until the end of the experiment, the pans were watered as needed with distilled water. In figure   the

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[4] 2 gm., $NaNO_3$; 1 gm., KH_2PO_4; 0.5 gm., KCl; 0.5 gm., $MgSO_4$; 0.01 gm., $FeSO_4$; 20 gm., dextrose; 15 gm., malt extract; 20 gm., agar; 1,000 cc., water.
[5] 2 gm., $NaNO_3$; 1 gm., KH_2PO_4; 0.5 gm., KCl; 0.05 gm., $MgSO_4$; 0.01 gm., $FeSO_4$; 50 gm., dextrose; 15 gm., malt extract; 1,000 cc., water.
[6] The formula used was as follows: $Ca(NO_3)_2$, 0.005M; KNO_3, 0.005M; $MgSO_4$, 0.002M; KH_2PO_4, 0.001M; NaCl, 0.001M; traces of H_3BO_3, $CuCl_2$, $ZnCl_2$, $MnCl_2$, $FeCl_3$.

FIGURE 2.—Sand-culture pans arranged in a series of Wisconsin soil-temperature tanks.

general arrangement of the culture pans in the soil-temperature tanks and the siphon in the corner of each pan are shown.

The seedlings were carefully removed from the sand 28 to 30 days after planting and graded as to severity of symptoms of pink root into five disease classes, each of which was given a class weight as follows: Free of pink root, 0; slightly affected, 25; moderately affected, 50; severely affected, 75; dead, 100. A disease index for each lot was obtained by multiplying the number of plants in each class by the class weight, adding the products, and dividing the sum by the total number of plants.

EXPERIMENTAL RESULTS

CONCENTRATION OF INOCULUM

Inoculum of isolate Utah–T of *Pyrenochaeta terrestris* was mixed at the rate of 12.5, 25, 50, 100, or 200 cc. to 10 kg. of sand. The inoculated substrate was leveled off to within 1.5 inches of the top of 6-inch crocks. Each concentration of inoculum was replicated four times at 16°, 20°, 24°, and 28° C. in Wisconsin soil-temperature tanks. Excel, Texas Grano, Mountain Danvers, and Yellow Globe Danvers varieties were planted at the rate of 30 seeds of each variety. Data were taken 28 days after planting (table 2).

TABLE 2.—*Concentration of inoculum of isolate Utah–T of Pyrenochaeta terrestris in relation to disease development on 4 onion varieties at 4 sand temperatures*

Variety	Concen-tration	Disease index at temperature indicated			
		16° C	20° C.	24° C.	28° C.
	Cc. per 10 kg				
Excel	12.5	0	3	16	24
	25.0	0	4	28	39
	50 0	1	10	27	72
	100.0	4	19	38	76
	200.0	5	20	40	71
Texas Grano	12.5	0	10	70	96
	25.0	2	36	89	96
	50.0	14	41	84	100
	100.0	5	28	80	99
	200.0	10	56	95	99
Mountain Danvers	12 5	0	18	87	100
	25.0	6	55	97	98
	50.0	14	49	93	100
	100.0	11	54	94	100
	200.0	16	51	96	100
Yellow Globe Danvers	12 5	0	3	67	96
	25.0	0	15	87	91
	50.0	5	23	85	98
	100.0	6	28	75	98
	200.0	8	28	89	100

The disease index was influenced by resistance of the host, the temperature of the substrate, and the concentration of the inoculum. At 16° C. the disease was so slight that the influences of variety and concentration of inoculum were not measurable. The indices were higher at 20° than at 16°. There was a steady increase of the index of the most resistant variety, Excel, with increase of inoculum, but the index curves of the other three varieties, which were obviously

more susceptible than Excel, leveled off at 50 cc. or less of inoculum. At the more favorable temperatures, 24° and 28°, the differential in index due to inoculum became very slight or nil except in Excel. At 28° the indices of this variety were lower at all concentrations than those of the other varieties, but concentrations of 50, 100, and 200 cc. of inoculum did not have appreciably different effects on Excel. It was concluded that the maximum index due to increase in concentration of inoculum was reached more promptly as the temperature increased and as the inherent resistance of the host decreased. The relations of concentration of inoculum and of temperature to the disease indices of the most resistant variety, Excel, and of one of the three more susceptible varieties, Yellow Globe Danvers, are shown graphically in figure 3.

VARIATION IN PATHOGENICITY OF ISOLATES

Inocula of isolates Utah–H, Mich–H, Iowa–H, Tex–H, Cal–B–H, and Cal–D–H were prepared in the usual manner, and 6-inch crocks of sand and of soil (two-thirds greenhouse loam, one-third muck soil) were inoculated. Five White Portugal sets, surface-sterilized in 1 : 1,000 mercuric chloride for 20 minutes, were planted in each crock. The crocks were then placed in soil-temperature tanks set at 25° C. Plants were pulled after 30 days and rated as to severity of infection (table 3). Isolates Utah–H, Tex–H, and Cal–D–H were highly pathogenic, isolates Cal–B–H and Iowa–H moderately so, and isolate Mich–H weakly pathogenic in both sand and soil. In general a lower disease rating was obtained in soil than in sand.

TABLE 3.—*Pathogenicity of 6 isolates of Pyrenochaeta terrestris on White Portugal onion sets in sand and soil at 25° C.*

Isolate	Disease index in—		Isolate	Disease index in—	
	Sand	Soil		Sand	Soil
Utah–H	100	100	Cal–B–H	40	5
Tex–H	100	90	Iowa–H	45	5
Cal–D–H	100	80	Mich–H	5	0

In another experiment a second group of isolates was tested on seedlings in sand culture in pans. Two rows each of Yellow Bermuda, the parent of Excel (table 2), and Southport Yellow Globe varieties were planted in each of two pans inoculated with each isolate. The isolates used and the disease indices obtained 28 days after planting at 24° C. are presented in table 4. The disease indices for Southport Yellow Globe ranged from 100 for isolate La–S to 44 for isolate Wis–M–T. The indices for Yellow Bermuda were lower than those for Southport Yellow Globe. For any isolate the differences between variety indices were highly significant. The descending order of the indices was not the same for Yellow Bermuda as for Southport Yellow Globe. However, isolates La–S and Tex–T were more virulent than the others and Wis–M–T was the least virulent on both varieties.
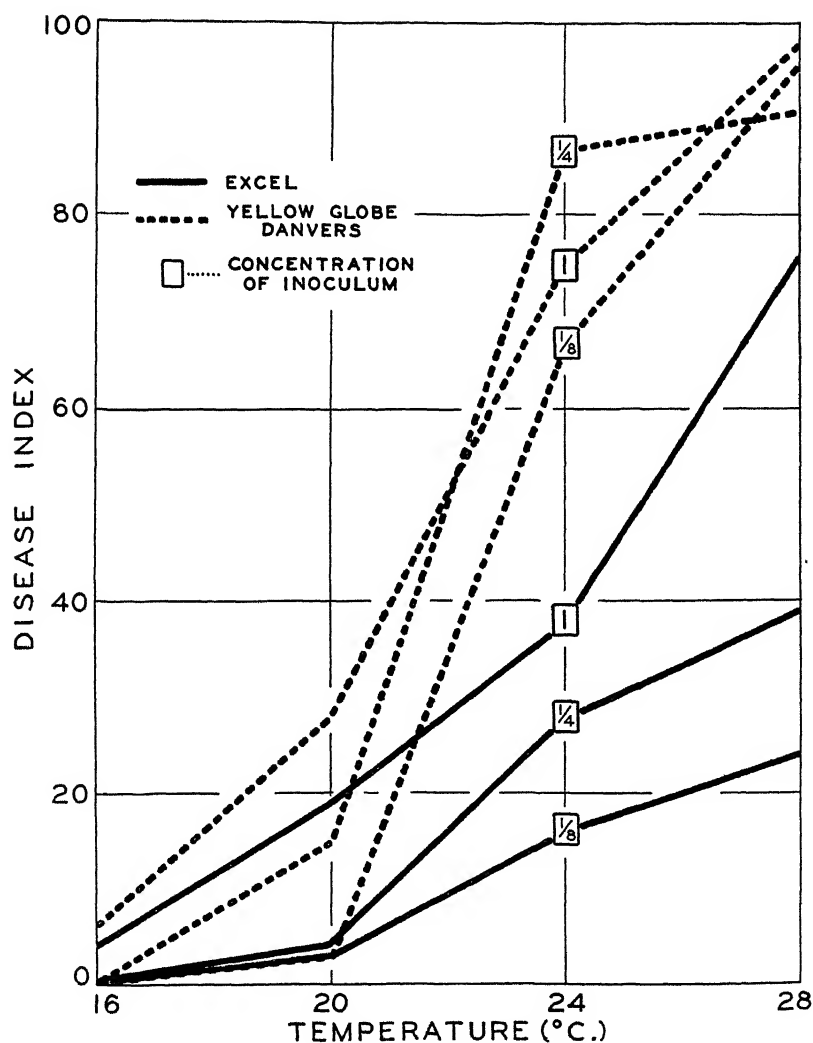
FIGURE 3.—Relation of temperature and concentration of inoculum to disease indices in seedlings of Excel and Yellow Globe Danvers onions. 1,100 cc. of inoculum to 10 kg. of sand; ¼, 25 cc.; ⅛, 12.5 cc.
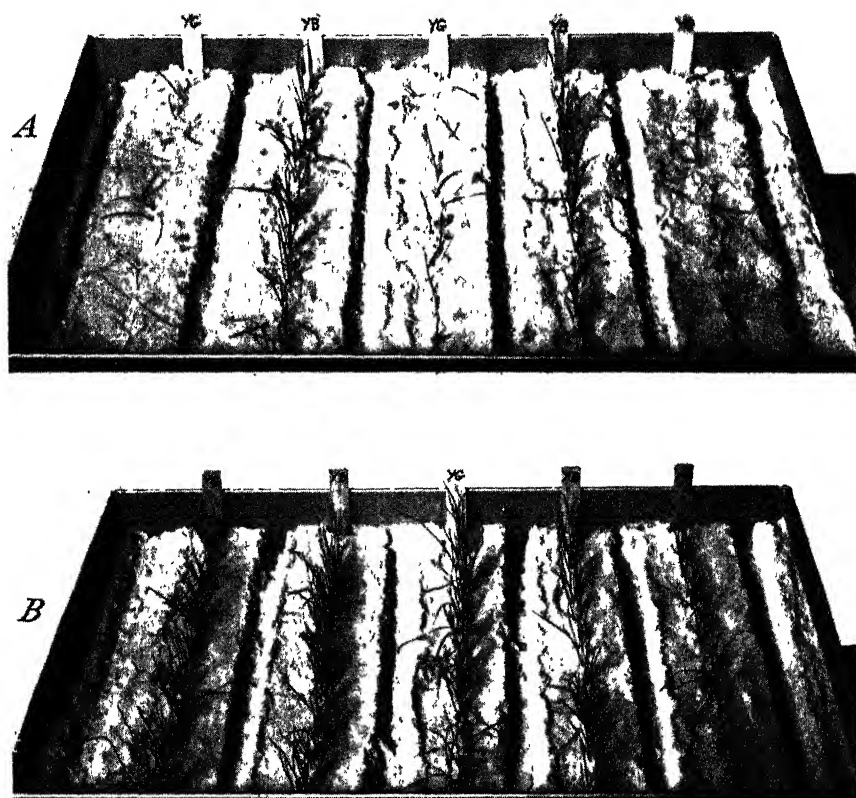
FIGURE 4.—Southport Yellow Globe (YG) and Yellow Bermuda (YB) plants grown for 28 days at 24° C. in sand inoculated with (*A*) a highly virulent isolate (La–S) and (*B*) a weakly virulent isolate (Col–FC–T). The greater resistance of Yellow Bermuda plants is more evident with isolate La-S.

TABLE 4.—*Pathogenicity of 8 isolates of Pyrenochaeta terrestris on seedlings of 2 onion varieties at 24° C.*[1]

Isolate	Disease index in variety—		Isolate mean
	Southport Yellow Globe	Yellow Bermuda	
La–S	100	90	95.0
Tex–T	99	91	95.0
Utah–T	88	48	68.0
Col–RF–T	86	39	62.5
Mass–T	76	64	70.0
Col–FCl–T	75	39	57.0
Iowa–T	73	48	60.5
Wis–M–T	44	29	36.5
Variety mean	80 1	56.0	

	5-percent level	1 percent level
[1] Least significant difference:		
Varieties	1.7	2.3
Isolates	3.5	4.6
Varieties × isolates	6.9	9.2
Within varieties or isolates	4.9	6.5

The comparative studies of isolates showed that rather wide differences in virulence existed. Other experiments, not presented in detail, showed that isolates from the same locality also varied in virulence. From the comparison of host varieties it appeared that the differences in host resistance were less pronounced when highly virulent isolates were used as the source of inoculum than when weakly virulent isolates were used. A comparison of Yellow Bermuda and Southport Yellow Globe varieties infected by a highly virulent isolate and a weakly virulent one is given in figure 4. Insofar as observations were conducted, cultures of individual isolates maintained by mass transfers on culture media about every 3 months retained about the same degree of virulence for 18 months.

TEMPERATURE RELATIONS

Hansen (*3*) found the optimum for growth of the pink root fungus on corn-meal agar to be about 26° C. Davis and Henderson (*1*) reported the optimum for growth on potato-dextrose agar to be 28°. In this investigation, plates containing 25 cc. of potato-dextrose agar were inoculated with 6-mm. disks from the borders of colonies on the same kind of agar. The isolates studied and the average diameters of colonies at 8 and 12 days are given in table 5. Six isolates made optimum growth at 28°, while two showed best growth at 24° on both the eighth and the twelfth day. Three (Utah-O-H, Mass-T, and Cal-D-T) made about as good growth at 24° as at 28°. The rates of increase of colony diameters with increases of temperature were not the same for all isolates. This is shown graphically in figure 5. The least growth at 28° was made by Mass-T and the most by Wis-R-T; the growth of Tex-1-H was intermediate.

TABLE 5.—*Relation of temperature to growth of 11 isolates of Pyrenochaeta terrestris on potato-dextrose agar*

Isolate	Diameter of colonies at temperature and days indicated [1]									
	16° C.		20° C.		24° C.		28° C.		32° C.	
	8	12	8	12	8	12	8	12	8	12
	Mm.	*Mm.*	*Mm.*	*Mm.*	*Mm.*	*Mm.*	*Mm.*	*Mm.*	*Mm.*	*Mm.*
Utah-O-H	17.5	31.8	29.5	41 5	33 5	56.5	33.0	58 0	16.8	35.3
La-M-T	14.3	29.5	28.5	55 8	45.8	78.8	47.8	90.0	24.8	44.8
Ill-T	17.0	34 0	32.0	55.8	45.3	71.8	40.0	64.0	22.0	43.0
La-S-S	14 3	27.5	25.0	49.0	47.0	78.8	42 5	72.5	21 0	41.0
Iowa-T	14 5	26.3	25.3	46.0	44 0	71.0	51.0	90.0	19.8	33.5
Col-FC-H	11.5	27.0	23.0	49 5	44.8	76 0	48.5	90.0	29.5	46.5
Tex-1-H	17.0	26.5	27.8	48.8	37.5	63.3	43.8	68.5	6.3	10.0
Wis-R-T	11.8	20.5	31.0	54.5	48.5	84 0	55.8	90.0	15.0	19.0
Wis-M-T	18.8	29.0	37.3	59.8	46.0	72.0	53.0	72.3	16.5	25.3
Mass-T	11.0	20.5	25.3	44.5	30.8	44.5	28 0	51.5	2.0	3.8
Cal-D-T	12.5	27.0	25.5	49.7	46.5	76.8	47.5	71.3	31.3	48.0

[1] At a reading of 90.0 the plate was completely occupied by the culture.

The relation of temperature to development of pink root was investigated by Hansen (*3*). Hansen planted onion bulbs in inoculated steam-sterilized soil in pots which were placed at 4° C., 10°, 13°, 20°, 25°, or 30° in the dark. He found that all of the 20 plants exposed
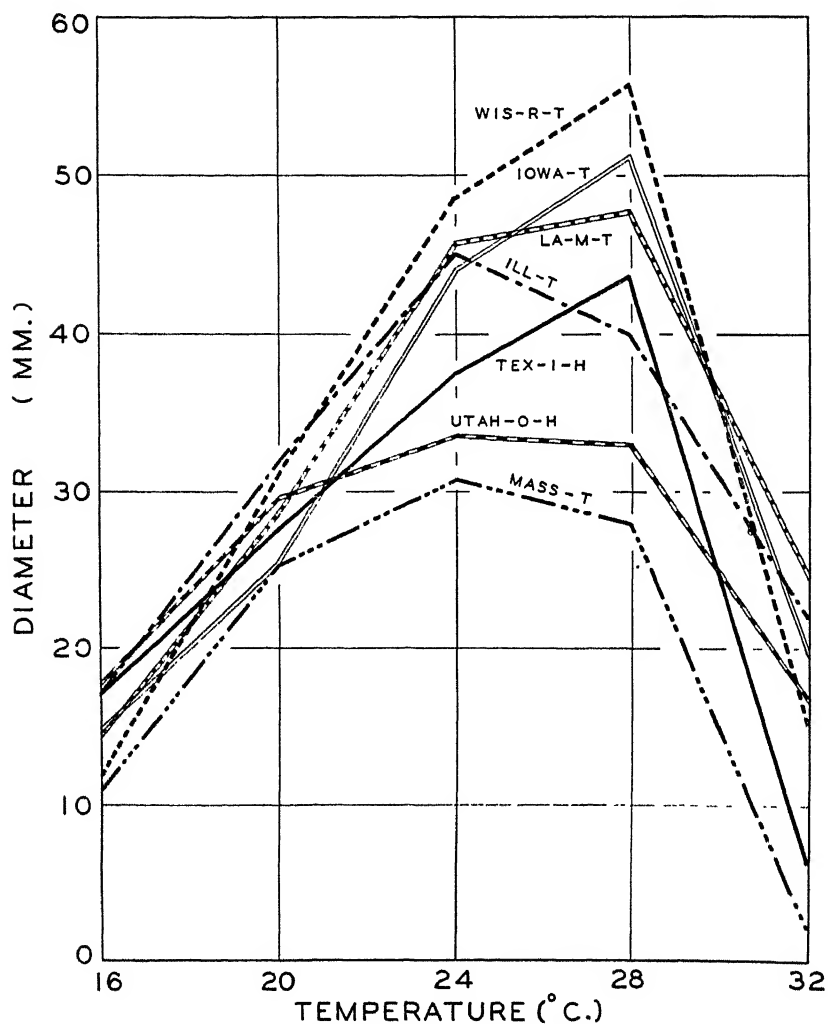
FIGURE 5.—Relation of temperature to diameter of colony of isolates Wis–R–T, Iowa–T, La–M–T, Ill–T, Tex–1–H, Utah–O–H, and Mass–T of *Pyrenochaeta terrestris* after 8 days.

to 25° were infected, whereas only 4 were infected at 30° and 15 at 20°. The best plant development occurred at 25°. Hansen concluded that the optimum for disease development was not far from 26°. The results of the present investigation (table 2) show that the index of isolate Utah–T increased with temperature up to 28°. In another experiment 4 varieties were grown in sand inoculated with isolates Tex–1–T, La–S–S, Iowa–S, and Wis–R–T at 16°, 20°, 24°, and 28°, respectively. The results are presented in table 6.

TABLE 6.—*Relation of 4 isolates of Pyrenochaeta terrestris and sand temperature to disease development* [1]

| Variety | Isolate | Disease index at temperature indicated | | | | Variety mean |
		16° C.	20° C.	24° C.	28° C.	
Excel	Tex–1–T	79	93	99	100	78.5
	La–S–S	51'	67	84	94	
	Iowa–S	38	73	93	97	
	Wis–R–T	35	70	90	94	
Texas Grano	Tex–1–T	84	99	100	100	74.0
	La–S–S	79	95	98	100	
	Iowa–S	25	41	76	94	
	Wis–R–T	23	30	59	83	
Mountain Danvers	Tex–1–T	76	97	99	100	67.8
	La–S–S	66	83	91	100	
	Iowa–S	23	35	50	82	
	Wis–R–T	21	28	57	78	
Yellow Globe Danvers	Tex–1–T	71	95	100	100	64.2
	La–S–S	65	84	90	98	
	Iowa–S	20	33	54	76	
	Wis–R–T	16	24	34	67	
Temperature mean		48.3	65.4	79.6	91.4	
Isolate mean (all varieties and all temperatures).	Tex–1–T	93.3				
	La–S–S	84.1				
	Iowa–S	56.9				
	Wis–R–T	50.6				

	5-percent level	1-percent level
[1] Least significant difference:		
Varieties	2.7	3.5
Isolates	2.7	3.5
Temperatures	2 7	3.5
Within varieties, isolates, or temperatures	10 4	14.1

Isolate Tex–1–T was much more virulent than isolates Iowa–S and Wis–R–T and slightly more virulent than isolate La–S–S. The differences were more pronounced at 16° C. and usually became less so at each step increase in temperature. The most severe disease development was at 28°. Excel apparently was as susceptible to isolate Tex–1–T as Texas Grano, Mountain Danvers, and Yellow Globe Danvers. It was more susceptible than the last two varieties to isolates Wis–R–T and Iowa–S.

HYDROGEN-ION RELATIONS

Davis and Henderson (*1*) reported a pH range of 4.2 to 7.4 to be favorable to growth of the pink root fungus on potato-dextrose agar. In the present investigation growth was studied on a modified Hoagland's nutrient solution [7] containing 15 percent malt extract, 10 per-

[7] See footnote 6, p. 3

cent dextrose, and 20 percent agar. Into each plate after sterilization 20 cc. of the medium was measured and adjusted to pH 4, 6, or 8 with sodium hydroxide or hydrochloric acid. Five replicates of each were inoculated with isolates La–S–S, La–M–T, Utah–O–H, and Tex-1--T and incubated at 24° C. for 8 days. The measurements recorded in table 7 were nearly the same at all pH levels for three isolates; for the fourth isolate (Utah–O-H) there was a small decrease in growth from pH 8 to pH 4.

In a second experiment isolate La–M–T was grown at pH 2, 3, 4, 5, 6, 7, and 8, respectively. There was no difference in growth at pH 4 to 8; a decided retardation occurred at pH 3; and no growth occurred at pH 2.

TABLE 7.—*Growth of 4 isolates of Pyrenochaeta terrestris on malt-extract agar of 3 initial pH levels*

Isolate	Diameter of colony at initial pH level indicated		
	4	6	8
	Millimeters	*Millimeters*	*Millimeters*
La–M–T	54.6	54.4	55.2
La–S–S	46.8	46.2	44.8
Tex–1–T	53.6	55.6	55.4
Utah–O–H	48.8	52.2	56.0

Kreutzer (*4*) studied the nature of the influence of hydrogen-ion concentration on the manifestation of color in onion roots infected by the pink root fungus. He placed diseased roots in a liquid and changed the pH of the latter. The color changed from red or red purple at pH 8.5 to yellow brown at pH 4.5. In the present investigation four varieties of onion were grown in sand inoculated with four isolates and placed in a continuous-drip nutrition system (*6*) in which the nutrient was adjusted to pH 4, 6, and 8, respectively. The nutrient solutions at the two lower pH levels had changed very slightly when they left the sand culture. The pH 8 nutrient had shifted to about pH 7 when it left the pot. The disease indices are given in table 8. Isolate Utah–O–H was the least virulent and Tex–1–T the most virulent. Excel was consistently more resistant at all pH levels and to all isolates. There was no consistent difference in disease indices at the different pH levels. Root color varied with variety and isolate at each pH level. The darkest red roots were at pH 8; many brownish pink-roots were found at pH 4.

The heights of inoculated and uninoculated plants were recorded at the end of the experiment (table 9). There was no appreciable difference between plants at the three pH levels in the uninoculated sand. In the inoculated sand there was an inverse relation between height of plants and virulence of isolate, the greatest difference being between plants infected by isolate Utah–O–H and those by isolate Tex–1–T. Within a given isolate there was an inverse relation between disease index and plant height. The widest difference was that between Excel, the most resistant, and White Grano, the most susceptible variety. The difference was more pronounced with the three highly virulent isolates than with isolate Utah-O-H.

TABLE 8.—*Effect of initial pH on disease development on 4 varieties of onion inoculated with 4 isolates of Pyrenochaeta terrestris* [1]

Isolate	Variety	Disease index at initial pH level indicated			Mean for all pH levels	Isolate mean
		4	6	8		
Utah-O-H	Excel	5	15	10	10 0	20. 8
	White Grano	20	35	35	30 0	
	White Sweet Spanish	15	25	25	21. 7	
	Yellow Globe Danvers	15	25	25	21 7	
La-M-T	Excel	60	40	45	48 3	75. 4
	White Grano	85	90	90	88 3	
	White Sweet Spanish	80	70	85	78. 3	
	Yellow Globe Danvers	80	90	90	86. 7	
La-S-S	Excel	30	30	40	33. 3	70. 4
	White Grano	80	85	90	85. 0	
	White Sweet Spanish	80	80	90	83. 3	
	Yellow Globe Danvers	75	80	85	80 0	
Tex-1-T	Excel	50	50	45	48. 3	83 3
	White Grano	95	95	95	95 0	
	White Sweet Spanish	95	95	95	95. 0	
	Yellow Globe Danvers	95	95	95	95. 0	
Variety mean (all isolates and all pH levels).	Excel	35. 0				
	White Grano	74. 6				
	White Sweet Spanish	69 6				
	Yellow Globe Danvers	70. 8				

[1] Least significant difference:	5-percent level	1-percent level
Varieties	5. 3	7. 0
Isolates	5 3	7. 0
Varieties × isolates	14 9	19. 7

TABLE 9.—*Effect of initial pH on the height of seedlings of 4 varieties of onion inoculated with 4 isolates of Pyrenochaeta terrestris* [1]

Control or isolate	Variety	Height of seedlings at—			Mean for all pH levels	Control or isolate mean
		pH 4	pH 6	pH 8		
		Inches	*Inches*	*Inches*	*Inches*	*Inches*
Control (uninoculated)	Excel	8 5	8 0	9.0	8.4	8. 4
	White Grano	8. 5	8 0	8 5	8.3	
	White Sweet Spanish	8 0	7 5	8 0	8.1	
	Yellow Globe Danvers	9 0	8.5	9.0	8.8	
Utah-O-H	Excel	7 0	7.0	7.5	7.1	6. 9
	White Grano	6 5	7 0	7.0	6.7	
	White Sweet Spanish	6. 5	6. 5	6.5	6 5	
	Yellow Globe Danvers	7. 5	7 0	7 5	7.3	
La-M-T	Excel	4. 5	5 5	5.0	5 0	3. 3
	White Grano	2 0	3.0	1.5	2 3	
	White Sweet Spanish	3. 5	4 0	2 5	3. 4	
	Yellow Globe Danvers	2 5	3.0	2.0	2 6	
La-S-S	Excel	5 0	5.5	5 5	5 3	3. 8
	White Grano	2. 0	3.5	3.0	2 8	
	White Sweet Spanish	3. 0	3.5	3 0	3. 3	
	Yellow Globe Danvers	4 0	4 0	3.0	3 8	
Tex-1-T	Excel	4. 0	5 0	4 5	4. 5	3. 2
	White Grano	2. 0	3 0	3 0	2.8	
	White Sweet Spanish	3. 0	3 5	3.0	3.1	
	Yellow Globe Danvers	2. 5	2. 5	2.5	2.5	
Variety mean (all isolates and all pH levels).	Excel	5. 5				
	White Grano	3 7				
	White Sweet Spanish	4. 1				
	Yellow Globe Danvers	4. 1				

[1] Least significant difference:	5-percent level	1-percent level
Varieties	0. 4	0. 5
Isolates	. 4	. 6

HOST RESISTANCE

Taubenhaus and Mally (*8*) reported that in Texas the Bermuda varieties of onion were moderately resistant to pink root, whereas other varieties tested, including Sweet Spanish, were very susceptible. Porter and Jones (*5*) found that in California Australian Brown and Yellow Globe Danvers were susceptible and Sweet Spanish only moderately so. In the present investigation results from the experiments already reported showed a difference in degree of pink root development in varieties grown for 28 days on heavily infested sand at temperatures very favorable for the causal fungus. In most instances Yellow Bermuda and Excel showed lower indices than other varieties tested. This is in accord with the reputed behavior of Bermuda varieties in infested soil in the Rio Grande Valley and elsewhere. The experiments also showed that the expression of resistance was suppressed as the temperature rose to 28° C. and in proportion to the virulence and concentration of the pathogen. Under optimum conditions, then, Yellow Bermuda eventually showed a disease index of 100.

The optimum conditions for severe seedling tests having been worked out, various onion varieties were tested for susceptibility to a large number of isolates. The data from the first series are given in table 10. The disease indices of isolates Mass–T, Col–FC1–T, Wis–R–T, and Wis–M–T averaged highly significantly lower than those of the other isolates. Yellow Bermuda–1 had a highly significantly lower index than any other variety tested, and this difference was usually significant when varieties were compared within each individual isolate. Excel–1 was the next in resistance, but its index was not so consistently low as that of Yellow Bermuda–1.

TABLE 10.—*Reaction of onion varieties to isolates of Pyrenochaeta terrestris, series 1* [1]

Variety	Disease index of isolate indicated										Variety mean
	Cal-D-T	La-S	Ill-T	Tex-1-S	Col-FC1-T	Iowa-S	Utah-T	Mass-T	Wis-M-T	Wis-R-T	
Nebuka	96	81	98	55	38	95	64	44	77	62	71.0
Yellow Bermuda-1	71	72	83	62	29	80	63	29	54	37	58.0
Excel-1	96	75	94	61	33	96	70	37	56	74	69.2
Brigham Yellow Globe-1	96	86	81	86	55	78	84	43	56	54	71.9
Brigham Yellow Globe-2	95	88	86	86	57	81	91	44	66	48	74.2
Red Wethersfield-1	95	88	83	83	50	82	89	41	70	48	72.9
Yellow Globe Danvers-1	98	87	86	82	53	83	89	45	64	54	74.1
Yellow Globe Danvers-2	98	89	87	86	58	87	89	50	72	52	76.8
Early Yellow Globe-1	99	90	85	90	62	85	96	46	67	56	77.6
Ebenezer-1	96	94	83	87	56	86	89	43	67	55	75.6
Sweet Spanish-1	97	88	89	79	46	88	89	58	76	58	76.8
White Sweet Spanish-1	95	92	85	80	52	82	89	50	74	57	75.6
Australian Brown-1	98	92	94	86	52	89	93	44	74	55	77.7
Sutton's A1	98	95	91	88	56	92	89	55	69	60	79.3
Ailsa Craig	100	100	100	97	69	99	96	75	80	76	89.2
Autumn Queen	98	95	100	88	70	96	92	73	89	84	88.5
Isolate mean	95.4	88.3	89.1	81.0	52.3	87.4	85.8	48.6	69.4	58.1	

[1] Least significant difference:

	5-percent level	1-percent level
Varieties	3.4	4.5
Isolates	2.7	3.6
Varieties × isolates	15.2	20.1
Within varieties or isolates	10.7	14.2

The data from the second series are given in table 11. The disease indices of isolates Cal–D–T, La–S–S, Ill–T, and Col–FC2–T averaged significantly higher than those of the other isolates. Beltsville Bunching (an amphidiploid) was the most resistant variety, followed by Excel–2 and California Hybrid Red. Excel–2 was grown from a fresh lot of seed, whereas Excel–1 was grown from seed of the previous year. Thus the slightly higher disease index of Excel–1 may have been due partly to less vigorous growth after germination. All the other varieties tested were uniformly susceptible.

TABLE 11.—*Reaction of onion varieties to isolates of Pyrenochaeta terrestris, series 2* [1]

| Variety | Disease index of isolate indicated | | | | | | | | | | Variety mean |
	Cal–D–T	La–S–S	Ill–T	Tex–1–S	Col–FC2–T	Iowa–1–T	Utah–T	Mass–T	Wis–M–T	Wis–R–T	
Beltsville Bunching	99	100	94	35	99	35	41	62	87	77	72.9
Excel–1	97	100	97	69	99	76	65	73	94	97	86.7
Excel–2	98	98	99	54	98	73	57	70	97	91	83.5
Brigham Yellow Globe–1	100	100	96	81	100	76	94	77	93	89	90 6
Brigham Yellow Globe–3	100	100	91	92	100	82	94	84	92	91	92.9
Red Wethersfield–2	100	100	96	78	100	77	90	79	92	91	90 3
Yellow Globe Danvers–1	100	100	99	82	100	76	94	77	91	87	90. 6
Yellow Globe Danvers–2	100	100	99	81	100	83	96	81	95	91	92 6
Early Yellow Globe–1	100	100	98	91	100	72	97	75	91	89	91.3
Ebenezer–1	100	100	97	80	100	70	91	71	90	89	88. 8
Sweet Spanish–1	100	100	98	73	100	80	85	75	98	95	90. 4
White Sweet Spanish–1	100	100	99	79	100	77	94	77	96	92	91 4
Australian Brown–1	100	100	100	73	100	80	90	85	95	98	92.1
California Hybrid Red	100	100	95	82	100	61	93	67	72	78	84.8
Texas Grano	100	100	98	82	100	69	96	75	96	87	90.3
Isolate mean	99. 6	99. 9	97.3	75. 5	99 7	72. 5	85 1	75. 2	91.9	89.5	------

	5-percent level	1-percent level
[1] Least significant difference:		
Varieties	2.4	3.2
Isolates	1.9	2.5
Varieties × isolates	10.7	14.1
Within varieties or isolates	7.6	10.0

The data from the third series are presented in table 12. Again the disease indices of isolates Cal–D–T, La–S–S, and Col–FC2–T were much higher than those of the other isolates. Yellow Bermuda–2 and White Sweet Spanish–2 were significantly more resistant than the other varieties. Crystal Grano was significantly more susceptible than the other varieties.

A comparison of disease indices of sand- and soil-culture series is given in table 13. The indices for the sand-culture series represent an average of all varieties inoculated with a given isolate. It can be seen that there was a general tendency for the indices of the soil series to be lower than those of the sand series. There was also more variation in the soil-culture series. Isolates Cal–D–T, La–S–S, and Col–FC2–T gave consistently high indices in both soil and sand culture. The reaction of the solutions siphoned from the pans was approximately pH 7.3. There was no significant difference between the reaction of solutions from pans inoculated with the various isolates.

TABLE 12.—*Reaction of onion varieties to isolates of Pyrenochaeta terrestris, series 3* [1][2]

Variety	Disease index of isolate indicated										Variety mean[1]
	Cal-D-T[1]	La-S-S[1]	Ill-T[1]	Tex-2-S	Col-FC2-T[1]	Iowa-S	Utah-T	Mass-T	Wis-M-T	Wis-R-T	
Yellow Bermuda-2	95	90	63	30	100	80	40	60	73	65	58.0
White Sweet Spanish-2	94	93	70	50	100	80	60	65	65	18	61.3
White Sweet Spanish-3	96	96	93	65	100	85	65	95	99	85	82.3
Sweet Spanish-2	99	100	85	55	100	89	75	90	88	80	79.5
California Early Red	100	95	95	75	100	73	93	70	75	65	75.2
Crystal Wax	98	93	83	55	100	86	65	83	73	60	70.3
Australian Brown-2	100	90	80	60	100	80	90	60	70	60	70.0
Lord Howe Island	99	100	83	55	100	92	75	70	65	70	71.2
Southport Red Globe	98	99	75	55	100	88	85	68	80	63	73.2
Yellow Globe Danvers-3	100	100	80	60	100	90	85	75	63	75	74.7
Ebenezer-2	100	100	83	50	100	91	90	78	75	65	74.8
White Babosa	100	100	90	55	100	95	80	80	85	65	76.7
Early Yellow Globe-2	100	100	83	55	100	94	98	88	80	70	80.8
White Grano	100	100	83	70	100	92	95	80	83	73	82.2
White Portugal	100	98	90	75	100	90	90	95	95	80	87.5
Crystal Grano	100	100	100	80	100	94	100	99	99	93	94.2
Isolate mean	98.7	97.1	83.5	59.1	100.0	87.4	80.4	78.5	79.3	69.8	

[1] Disease-index values for isolates Cal-D-T, La-S-S, Ill-T, and Col-FC2-T not used in the determination of variety means and in the statistical analysis.

[2] Least significant difference:

	5-percent level	1-percent level
Varieties	5.9	7.8
Isolates	3.6	4.8
Varieties × isolates	20.5	26.9
Within varieties or isolates	14.5	19.1

TABLE 13.—*Disease indices in unsterilized soil and in sand inoculated with various isolates of Pyrenochaeta terrestris*

Isolate	Disease index in series and substrate indicated					
	Series 1		Series 2		Series 3	
	Sand	Soil	Sand	Soil	Sand	Soil
Cal-D-T	95	90	99	88	99	78
Utah-T	86	10	84	25	80	0
Ill-T	89	70	95	55	84	5
Wis-M-T	69	50	90	80	79	10
Wis-R-T	58	65	88	60	70	40
Mass-T	49	60	75	30	80	10
La-S	88	65	--	--		
La-S-S			100	100	97	80
Col-FC1-T	52	90				
Col-FC2-T			100	100	100	95
Tex-1-S	81	25	75	60		
Tex-2-S					59	1
Iowa-S	87	35			87	5
Iowa-1-T			72	50		

DISCUSSION

One of the objects of the present investigation was to develop a standardized technique whereby large numbers of individual onion plants could be tested for their resistance or susceptibility to *Pyrenochaeta terrestris*. The experimental results show that highly uniform and reproducible results can be obtained by using inoculated white

quartz sand as a substrate, in which the complex introduced by soil flora and other soil factors is removed.   By the use of Yellow Bermuda, the most resistant variety in field tests, as a standard, it was demonstrated that an index of resistance could be obtained on seedlings in about 28 days.   The expression of resistance, however, was influenced by a number of environmental factors; in fact conditions were created in which Yellow Bermuda was completely killed.   The severity of disease, as measured by the index described, increased up to a certain point with increase in concentration of inoculum and in the temperature maintained in the sand.   Isolates from various parts of the United States and those from within a given area varied in degree of virulence.   In general, isolates carried in culture by mass transfer maintained about the same degree of virulence over a period of 18 months; there was little indication that they differed greatly in their selective pathogenicity to the varieties studied.

Twenty-three standard varieties were tested by mixing 100 cc. of standardized inoculum with 10 kg. of sand and growing seedlings therein for 28 days. All seedlings, even those of the Yellow Bermuda–2, were killed promptly by the most virulent isolate (Col–FC2–T, table 12).   When isolates of intermediate virulence (Utah–T and Ill–T, table 12) were used a large percentage of the Yellow Bermuda–2 plants survived, but all plants of Crystal Grano, the most susceptible variety, succumbed.   Under the controlled conditions just described an isolate of intermediate virulence was used satisfactorily to screen several hundred breeding progenies from which the most resistant segregates had to be selected.   Obviously an extremely virulent pathogen might have been selected as a standard, and the rate of disease development might have been retarded by reducing the concentration of the inoculum or the sand temperature or both to provide conditions under which a certain percentage of the Yellow Bermuda standard would survive.   The point emphasized here is that by using the results of this investigation it was possible to set up a standard test in which environment and virulence of pathogen were controlled to the point at which screening tests from month to month and year to year were reasonably comparable; such a test could not be developed in the field.

SUMMARY

A large number of isolates of the pink root fungus (*Pyrenochaeta terrestris*) from various parts of the United States were assembled and studied.

The optimum temperature for growth of the various isolates was 24° or 28° C.   There were marked differences in rate of growth between isolates, especially at 28°.   They grew well at all hydrogen-ion levels tested within the range pH 4 to 8.   The isolates, even those from the same section, were found to differ widely in pathogenicity.

Maximum pink root development in sand culture occurred at 28° C.   Disease development in sand cultures to which the nutrient solutions were added by the use of a drip system was not affected by a change in pH within the range pH 4 to 8.

A pan, sand-culture method was developed. By the use of standardized inoculation procedure and controlled environment uniform infection of large numbers of seedlings could be obtained. By this method a survey was made of the resistance of 23 commercial varieties of onion to various isolates of *Pyrenochaeta terrestris* from widely separated areas. Yellow Bermuda and Beltsville Bunching were the most resistant varieties tested. Excel, a selection from Yellow Bermuda, approached but did not equal Yellow Bermuda in resistance.

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BACTERIOLOGICAL CHANGES DURING THE FERMENTATION OF STEAMED POTATOES FOR SILAGE[1]

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INTRODUCTION

During the summer of 1944 the War Food Administration sponsored experimental work at a number of State agricultural experiment stations on the utilization of surplus potatoes (*Solanum tuberosum* L.) for stock feed. The use of the surplus in the form of silage for livestock was stressed, and general recommendations as to the procedures to be followed were given. [3] It was stated that the best method was that of steaming and ensiling in pits.

The experimental studies at the North Carolina station were based on three carloads of potatoes and involved the cooperative efforts of several departments (Agricultural Engineering, Animal Industry, and Horticulture) as well as the Bureau of Agricultural and Industrial Chemistry of the United States Department of Agriculture. The joint work was planned to cover two general phases, namely, bacteriological and chemical changes during the fermentation and feeding trials on the ensiled material.

This report deals with the principal bacteriological changes occurring during the fermentation of the steamed potatoes. Such studies are of considerable interest in view of the nature of the preparation of this type of silage which calls for steaming the potatoes until cooked and promptly ensiling (while hot) relatively large tonnages in pits. Such a procedure would naturally introduce the question of the effect of prolonged high temperature upon the micro-organisms associated with normal silage fermentations, especially the lactic acid bacteria (of the *Lactobacillus* genus) and other non-heat-resistant types, such as the yeasts and coliforms. While the preparation and use of steamed potato silage has been the subject of investigation for many years, principally abroad, the bacteriological changes have not been clearly indicated.

PROCEDURE

Forty-five tons of U. S. No. 1, size B, potatoes were available for the work. The potatoes were shipped in carload lots from the eastern part of North Carolina and arrived in good condition. No attempt was made to remove the few rotten potatoes occasionally found. Thirty tons were used for the steamed-silage study and 15 tons were ensiled raw. Only the work on the steamed silage is discussed in this paper.
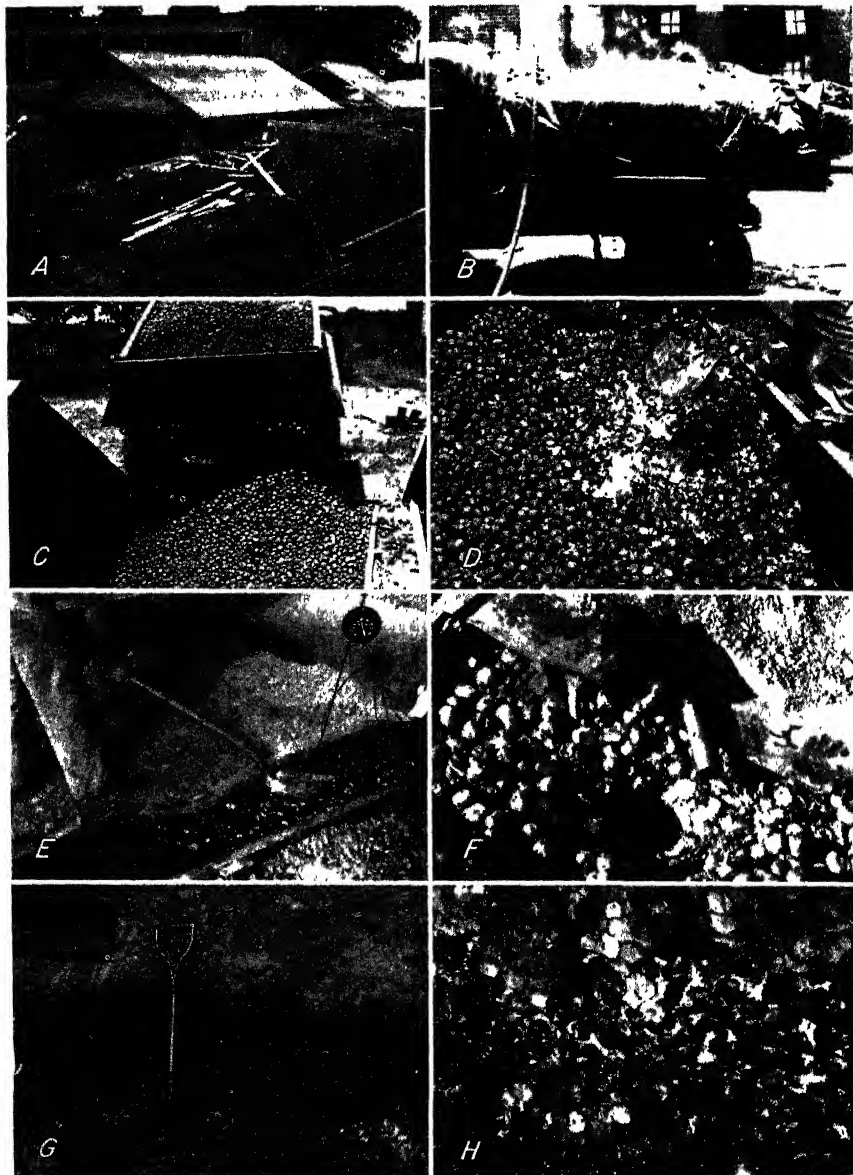
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EXPERIMENTAL SILO

The experimental trench silo used was one of two constructed at the Central Experiment Station farm near the college. These silos had approximately the following dimensions: 6 feet deep; 8 feet wide at the bottom, 11½ feet wide at the top; and 25 feet long. They had concrete floors and plastered concrete walls 3 to 4 inches thick, reinforced with wire fencing. The ends were closed by the use of 2- × 6-inch planks, supported by 4- × 4-inch posts. Both silos were provided with covers and were constructed in a hillside to provide natural drainage (fig. 1, *A*).

STEAMING OPERATION

The potatoes were steamed on July 10 and 11 at the college, two steel-bodied dump trucks being used. These trucks had a capacity of from 1½ to 2 tons of raw potatoes. On the bottom of each truck body was fastened a steam rake consisting of four parallel lengths of perforated ½-inch pipe connected to a horizontal cross member to which a vertical inlet was attached. The rake was connected to the steam line by means of a steam hose and pipe. The steam pressure was 120 pounds per square inch.

The potatoes were dumped into the trucks directly from the railroad cars, covered with heavy tarpaulins, and steamed until cooked throughout (fig. 1, *B* and *D*). This required from ¾ to 1 hour with the equipment used. The potatoes were kept covered and taken immediately to the trench silo (about a 10-minute trip) and dumped (fig. 1, *C*). In this manner, the 30 tons of potatoes were handled in about 12 working hours, using two trucks and two sources of steam.

FILLING THE SILO

The potatoes were dumped into the silo at three locations along one side. When all the steamed potatoes were in the trench, they were leveled off and covered with lapped strips of heavy tar-paper roofing (fig. 1, *E*). The mass was packed by carefully tamping the surface of the paper. A scant 4-inch layer of soil was then added for weighting and sealing. The 30 tons of steamed potatoes filled the trench to a depth of 3½ to 4 feet. About 30 hours after the first load had been steamed and dumped and about 6 hours after the last load had been put in the silo the temperature at the approximate center of the mass was in the range of 160°–164° F. Subsequent temperature readings were taken at two

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FIGURE 1.—Ensiling steamed potatoes. *A*, Experimental trench silo with three-piece cover, one of which (background) has been removed preparatory to dumping in the potatoes. *B*, Steaming the covered potatoes in a metal-bodied dump truck. *C*, Dumping the steamed potatoes in the front (north) section of the trench. *D*, Steamed potatoes in the trench before leveling and covering; consistency of potatoes at this stage shown in area struck by the shovel. *E*, The filled trench at the time of the leveling and covering operation; the dial thermometer (right foreground) read approximately 160° F. at the conclusion of filling and covering. *F*, Sampling: A core of the top layer of surface potatoes removed with a petri-dish can (left) prior to taking the bacteriological sample with the smaller pipette can (right). *G*, Part of the end (south) section of the ensiled potatoes opened after 8 months' storage; the potatoes form a tightly packed mass beneath the light-colored soil layer. *H*, Close-up of ensiled potatoes after cutting through a small area near the top; cavities represent areas where individual potatoes fell away.

locations at about mid-depth in the mass. A copper-constantan thermocouple was placed at one location and the bulb of a vapor-actuated dial thermometer at the other.

Samples were taken for bacteriological and chemical examination in the following manner: First, the soil was carefully removed from an area of tar-paper roofing (about 18 inches square) with a garden hoe, and the surface was brushed clean with a whisk broom. Then a U-shaped cut (8–10 inches on a side) was made in the paper and the flap was turned back. A sterile, copper petri-dish can (4½ inches in diameter and 8¼ inches long) was inverted and pushed vertically into the mass, and an 8-inch core of the top layer of potatoes was thus removed and discarded (fig. 1, *F*). The actual sample was obtained by removing a second core of potatoes with a sterile, copper pipette can (2 inches in diameter and 12 inches long) starting from the center of the surface left by the first cut. This gave about a 1½-pound sample from the central layer about 1 to 2 feet beneath the surface. The temperature of each sample area was taken by means of a maximum-indicating thermometer inserted into the wall of the cut at the time the sample was removed. After the sample was taken, the potatoes from the discarded core were tamped back into the hole. The tar-paper flap was then folded back in place and a new square of tar-paper larger than the opening was placed over the surface and covered with soil, after which the location was recorded. The sampling was restricted to about one-half of the total silo (fig. 2). This section was found to be the hottest
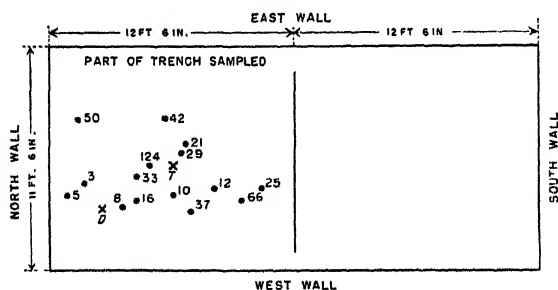
FIGURE 2.—Diagram representing top surface of the trench silo and indicating the comparative location of points at which core samples were taken for analysis. *D* marks location of the dial-type thermometer and *T*, the location of the thermocouple; the numbers indicate the age of the fermentation in days at each sampling.

after the filling operation was complete. Difference in temperature in opposite end portions of the ensiled mass was to be expected because of the method of filling the silo. This resulted in the space at the lower end of the silo being filled with cooler material, particularly after leveling.

After the sample was collected, it was taken promptly to the laboratory for analysis. Fifty grams of the potato mass was finely cut with a sterile spatula into a glass-top mason jar containing 450 grams of

sterile saline.   The 1:10 mixture was shaken 100 times by hand prior to making suitable dilutions for use with solid and liquid media.   Duplicate tests on 50-gram subsamples were run at each sampling interval. The remainder of the potato sample was reserved for chemical analysis by sealing it in glass jars and storing in a freezing cabinet.   The major chemical changes occuring during the fermentation are not given in this report, but the titratable acidity and pH values, based on examination of the 1:10 dilution used for bacteriological analysis, are included.

Bacteriological examination of the silage was made for various mesophilic and thermophilic groups of micro-organisms.   These are listed in table 1 together with the liquid or solid medium used and reference to the general procedure followed.   For the most part, the procedures for the mesophiles have been successfully used by the authors for a number of years in connection with brined vegetable and sweetpotato-vine-silage fermentations (*5, 6, 7, 8, 9, 10*).[4]   The methods of examination for the thermophilic groups are essentially those developed in the laboratories of the National Canners Association (*3, 4*).   It should be mentioned that in examinations for viable aerobic (*13*) and anaerobic spore-forms (*3*) by use of the media indicated, a boiled sample (2 minutes) of the 1:10 silage dilution was used which had been previously neutralized with sterile calcium carbonate.   The same was true for the H_2S-producing thermophilic anaerobes.   The counts for facultative thermophilic anaerobes are based on colonies resulting both from viable cells and spores from unboiled samples.

TABLE 1.—*Estimates of micro-organisms on potatoes (U. S. No. 1, B size) before and after steaming for silage*

Microbial group	Culture medium used and procedure reference	Count per milliliter of wash water [1]	
		Raw potatoes	Steamed potatoes [2]
Mesophiles (35° C incubation)·			
Total count_____	Nutritive caseinate agar+BCP [3]; plates (*10, 7*).	86, 000, 000	200
Lactic acid bacteria _____ _____	Nutritive caseinate agar+BCP; plates (*10, 7*).	[4] 0	0
Coliform group_____	Brilliant green agar; plates (*8, 9, 6*)____	25, 000, 000	0
Yeasts_____	Acidified dextrose agar; plates (*5, 10*)__	150, 000	0
Molds_____	Acidified dextrose agar; plates (*5, 10*)__	160, 000	0
Aerobic spore count___ _____	Nutritive caseinate agar+BCP; plates (*13*).	22, 000	100
Anaerobic spore count [5]_____	Liver broth+particles; tubes (*7*)_____	100	10
Thermophiles (55° incubation):			
Facultative anaerobes_____	Dextrose tryptone agar+BCP; plates (*3*).	3, 600	40
Non-H_2S-producing anaerobes___ __	Liver broth+particles; tubes (*3*)_____	10	10
H_2S-producing anaerobes_____	Sulfite agar; tubes (*4*)_____	10	10

[1] 450 grams of whole potatoes plus 450 grams of sterile saline shaken 100 times.
[2] Taken from a 1- to 1½-ton lot steamed 45 minutes to 1 hour in a steel-bodied dump truck covered with a heavy tarpaulin; steam pressure at the source was 120 pounds per square inch.
[3] Bromcresol purple; 0.04 gram per liter.
[4] Not detected because of predominance of and overgrowth by alkaline and coliform types.
[5] Includes putrefactive types as well as true butyric types of gas-forming, obligate anaerobes.

[4] Italic numbers in parentheses refer to Literature Cited, p. 31.

MICROSCOPIC COUNTS

At each sampling interval, 0.01-milliliter portions of the original 1:10 silage dilutions were placed in sequence on duplicate slides for microscopic counting. The smears were prepared and counted according to the method of Wang (*14*), a modification of the Breed (*2*) technique. One set of slides was stained with the Kopeloff and Cohen (*11*) modification of the Gram stain, and the number of individual Gram-positive cells in 100 fields per smear was determined. For the other set, the Schaeffer and Fulton modification (*12*) of the Wirtz (*15*) spore stain was used, and the number of individual spores in 100 fields per smear was noted. The microscopic counts for both cells and spores are reported in terms of millions per gram of silage.

TITRATABLE ACIDITY AND pH

As mentioned previously, titratable acidity and pH determinations were made on the original 1:10 dilution of the silage used for bacteriological purposes The pH determinations were made with the glass electrode. Titratable acidity was run on 10-milliliter aliquots of the 1:10 dilution (after allowing it to stand 1 to 2 hours) by titrating with 0.111 N NaOH using phenolphthalein as the indicator. The values were calculated in terms of grams lactic acid per 100 grams of potato silage. These determinations were made on the 1:10 dilution for the purpose of having available, at the time of plating, a reasonably clear picture of the progress of acid production during the fermentation.

RESULTS

EFFECT OF STEAMING ON SURFACE ORGANISMS

At the time the steaming operation was going on, an attempt was made to obtain an estimate of the relative number of different groups of organisms occurring on the surface of the uncut potatoes both before and after steaming. The results are given in table 1. It is evident that the steaming procedure greatly reduced the large number of surface organisms. The relatively few that survived were the heat-resistant, spore-forming types. As far as could be determined, the usual types of organisms associated with normal silage fermentations (i. e., lactic acid bacteria, yeasts, and coliforms) did not survive the heating. Although the initial count of the facultative anaerobic thermophiles was small in comparison with the total number of organisms present on the raw potatoes, the proportion of the thermophiles surviving the steaming operation was much greater. This is of considerable importance in view of their role in the subsequent fermentation of the ensiled potatoes.

TEMPERATURE CHANGES DURING FERMENTATION OF SILAGE

After the initial steaming operation, the surviving micro-organisms on the potatoes were subjected to further prolonged exposure to rather high temperatures during the time required for the hot mass to cool in the silo. Progressive temperature changes based on readings made repeatedly at two locations (fig. 2) in the silo are shown

in table 2. The location of each individual area sampled and the temperature of that particular area at the time of sampling are also presented. In general, the temperature of the mass 2 days after ensiling was in the range of 160° F. Furthermore, it remained above 120° for about the first 12 days and above 100° for the first 3 weeks. The dial thermometer location shows higher temperature readings during the first few days and lower readings after 12 days than the thermocouple location. This is accounted for by the fact that the thermometer was located where the steamed potatoes were dumped, which was the hottest area. However, it was also closer to the front and side walls, and therefore cooled faster than the more central location occupied by the thermocouple.

TABLE 2.—*Temperature changes during the fermentation of steamed potato silage*

Date (1944)	Age	Distance of area sampled from—		Temperature record			
		North wall	West wall	Sample area	Dial thermometer [1]	Thermocouple [2]	Atmosphere
	Days	*Inches*	*Inches*	° F.	° F	° F.	° F.
July 11, 8 p m	1					160	82
12, 2 30 p m	2				164	158	91
13, 8 a m	3	24	52	162	159	152	81
15, 2 30 p. m	5	12	46	136	145	140	86
18, 2:30 p m	8	46	38	132	131	132	77
20, 1 30 p. m	10	76	46	126	124	127	90
22, 2 30 p. m	12	102	51	122	118	122	82
26, 1·30 p. m	16	52	42	108	108	114	96
31, 2 p. m	21	82	76	104	101	108	87
Aug. 4, 1 30 p m	25	130	51	100	95	103	94
8, 1·30 p. m	29	80	72	100	92	100	76
12, 10 a. m	33	54	58	96	90	97	85
16, 10 a m	37	87	36	92	89	95	84
21, 10 a m	42	70	92	90	85	92	81
29, 11 a m	50	16	92	89	85	88	78
Sept. 14, 11 a. m	66	118	42	84	82	84	80
Nov. 11, 2 p. m	124	60	64		65	67	64

[1] Located 33 inches from the north wall and 33 inches from the west wall.
[2] Located 76 inches from the north wall and 66 inches from the west wall.

POPULATION OF MICRO-ORGANISMS DURING FERMENTATION OF SILAGE

The results of the bacteriological examination for the presence of various types of organisms during the silage fermentation are shown in table 3. Changes in acidity and pH of the ensiled potatoes as well as the approximate temperature of the core sample at each sampling interval are also given. The data indicate that the coliforms, yeasts, and lactic acid bacteria, groups usually associated with normal silage fermentation, did not contribute to the fermentation of this type of silage. This would seem reasonable as these groups are not heat-resistant and there was no evidence that they survived the steaming operation. Even if some few individual cells from these groups had survived the cooking, and some had been added during the filling operation, it is doubtful whether they would have withstood the elevated temperatures of the silage during the first 12 days.

TABLE 3.—*Populations of micro-organisms occurring during the fermentation of steamed potatoes for silage*

Sample date, 1944	Fermentation age	Temperature of sample	Thermophiles (55° C.), count per gram				Mesophiles (35° C.), count per gram					Microscopic count—individual cell and spore count per gram		pH	Titratable acidity as lactic acid
			Facultative anaerobes		Anaerobes[1]		Total count	Acid-forming colonies	Coliforms, yeasts, lactic acid bacteria, and molds[1]	Spore forms		Gram+ cells	Spores		
			Total count	Acid-forming colonies	Non-H2S-producing	H2S-producing				Aerobes	Anaerobes[1]				
	Days	*°F.*	*Millions*	*Millions*			*Millions*	*Millions*		*Hundreds*	*Hundreds*	*Millions*	*Millions*		*Percent*
July 13	3	162	0.093	0.093	<10		0.001	<0.001	0	0.95	0	2.8	1.8	5.50	0.40
15	5	136	.350	[2]0	<10	<10	.025	<.001	0	3.5	0	4.0	4.8	5.30	.50
18	8	132	1.9	1.9	1,000	<10	.041	<.001	0	2.0	0	4.0	6.6	4.70	.70
20	10	126	2.0	2.0	<10		2.13	2.13	0	<.1	0	15.2	9.0	4.78	.58
22	12	122	[3]5.1	5.1	100	<10	2.5	2.5	0	13.0	1	11.6	25.2	4.90	.48
26	16	108	2.8	2.8	100		22.5	22.5	0	4.5	0	19.2	6.4	4.20	.87
31	21	104	7.6	7.6	1,000		13.5	12.5	0	2.3	10	32.0	70.8	4.11	1.18
Aug. 4	25	100	1.9	1.9	100	<10	10.3	16.7	0	.6	0	14.2		4.60	.60
8	29	100	[5]11.5	11.5	<10		6.7	10.9	(4)	18.0	0	33.8	45.6	4.00	1.35
12	33	96		.01	<10		6.7		0	1.1	0	182.4	6.8	4.00	1.10
16	37	92	[5].25	.16	100		10.9	1.4	0	13.5	1	50.8	37.6	4.17	1.15
21	42	90	15.0	11.5	<10		1.4	[2]0	0	11.5	0	52.0	101.2	4.05	1.83
29	50	89	4.1	4.1	<10	<10	9.0	4.5	0	505.0	0	210.0	14.0	4.03	1.36
Sept. 14	66	84	2.7	2.7	<10		4.6	1	0	750.0	0	64.6	23.4	3.96	1.50
Nov. 11	124	67	1.0	.25	<10	<10	.27	>.01	0	19.0	1	210.4	63.8	3.95	3.40

[1] Zeros in column indicate less than 10 per gram.
[2] Not detected because of predominance of and overgrowth by alkaline types
[3] Spore count by plating boiled sample was 1.3 millions per gram.
[4] At this interval a count of 750 yeasts and 2,200 molds per gram was obtained, indicating probable contamination from the surface layer during sampling.
[5] Total count at 55° C. was 24 million per gram, using nutritive caseinate agar.

Examination of the silage with respect to certain of the anaerobic spoilage groups of bacteria, namely, the non-H_2S- and the H_2S-producing thermophiles, showed the former group to be present only in relatively small numbers on several occasions. The latter group appeared to be absent or was present to the extent of less than 10 per gram at the six times when examinations were made. Active growth by these two groups, if present, would have been expected during the 12-day interval when the potatoes were at or near the optimum range for thermophilic growth. Essentially the same results were obtained for the putrefactive anaerobes (mesophiles) including the true butyric and butylic types of gas-forming, obligate anaerobes. Here positive indication of the presence of spores was noted in only 5 of the 15 periodic samples and then only in relatively low numbers. The temperature of the potatoes during the first 12 days would be considered too high for growth of this group of anaerobes. After that time the reaction of the silage (about pH 4.5) would be unsuitable for growth. However, the test for growth of spore forms from boiled samples for certain members of this group of organisms is not necessarily a reliable index to their previous activity. Spore formation may be negligible prior to inhibition or death of the vegetative cells because of acid production in the presence of a readily fermentable carbohydrate. Nevertheless, active growth by these organisms in the silage would be associated with a malodorous fermentation. This condition was not found.

PREDOMINATING GROUPS OF MICRO-ORGANISMS

The previous discussion has dealt with several of the groups of microorganisms, both thermophilic and mesophilic, that appeared either inactive or absent during the fermentation proper. Further consideration of the bacteriological data in table 3 indicates that the thermophilic, facultative anaerobes were the predominating organisms during the fermentation and were responsible for the developed acidity and resultant decrease in pH of the ensiled mass. It will be noted that this group started activity rather soon after the potatoes were ensiled and for the most part showed populations in the millions per gram not only during the period when the mass was at elevated temperatures (120°–160° F.) but also when it was below the optimum temperature range for obligate thermophiles. The wide range of temperature tolerance accounts for the presence of rather similar plate counts obtained for this group listed in table 3 as mesophiles incubated at 35° C. (using nutritive caseinate agar). However, the 55° C. plate counts for the first three sampling periods, during the very early phase of the fermentation, indicate principally obligate thermophiles involved since comparatively few colonies were noted on the 35° C. plates at the same period. The facultative relationship toward temperature of the predominating types, after active fermentation was under way, was further demonstrated by the fact that, when the principal colonies from the 55° C. routine plates were transferred into liver broth and incubated at 35° C., growth occurred. The same was true for those picked from the 35° plates and incubated at 55°. Hence, so far as could be determined from the limited number of samples taken from the trench during the active acid fermentation, the predominating groups that grew out at either incubation temperature (55° or 35°) were essentially the

same.   There was one exception to this.   In certain instances during
the routine examinations, one specific colony type grew well at 35° on
nutritive caseinate agar, but very poorly, if at all, at 55° on dextrose
tryptone agar.   However, this was found to be a problem of nutritive
requirements concerning the two media used rather than temperature,
as will be discussed later.

(Legend on following page)

The routine plate counts during the fermentation period further showed that the major portion of the total count in most instances was composed of acid-forming colonies of the thermophilic, facultative anaerobic group. This was true for the plates incubated at either 35° or 55° C. These organisms brought about an acid fermentation that resulted in acidification of the ensiled mass and consequent preservation. The general course of acid development is shown by the lowering of the pH from about 5.5 at 3 days to about 3.95 at 50 days and thereafter until the conclusion of the sampling. During this period the titratable acidity increased from about 0.4 percent (calculated as lactic acid) to about 3.0 percent. The intensity of fermentation activity was not the same in all locations in the silo, as was indicated by the pH and titratable acidity values as well as by the irregular nature of some of the plate and spore counts. These counts did not always show typical, progressive populations. A greater lack of uniformity among samples would be expected in a more or less solid medium like the silage than in a fluid medium.

MICROSCOPIC COUNTS

The microscopic counts of individual Gram-positive cells give a reasonably good picture of the progress of the fermentation with respect to bacterial populations. In some ways it appears to give a better insight into the nature of this type of fermentation than do the cultural methods. Since fermentation activity did not always progress at the same rate in all locations in the ensiled mass, the sequence of microscopic fields was selected principally on the basis of the stage of fermentation of individual silage samples and not with strict regard to fermentation age of the particular sample (fig. 3).

The microscopic study revealed that the predominating flora was wholly Gram-positive and divided more or less into two morphological types; a short thick rod and a rather long rod of medium thickness (fig. 3, *C* and *D*). There is a possibility that a third type occurred, a much smaller rod than the others. Both principal types tended to elongate and form long chains and filaments in the more acid samples taken during the latter part of the fermentation (fig. 3, *G*). Predominating colonies picked from the routine platings likewise fell into the above morphological groups and showed the same tendency toward elongation in old cultures of broth media that had reached maximum acid production.

FIGURE 3.—Photomicrographs of the predominating micro-organisms present during the acid fermentation of steamed potato silage. All ×1700. Slides used were those prepared and stained for the microscopic cell counts of Gram-positive cells. *A, B,* Vegetative cells from samples taken during the early stage (10 and 16 days) of fermentation; dark area in background of *B* is a particle of steamed potato tissue. *C, D,* Samples taken at 33 and 50 days; both showing active acid fermentation with large populations of short, thick vegetative rods and a few of the more slender types. *E, F,* Samples taken at 21 and 42 days showing more advanced stage of fermentation and evidence of active spore formation; note clostridial form (*E, a*) and free, oval type spores (*F, a*). The background in each case (*E, F*) is composed of indistinct dead cells. *G,* Elongated cell type that predominated during the latter part of the fermentation; note definite swollen condition of one end (*a*) of the cell. Such cells gave rise to small spherical spores rather than to the large oval types shown in *F, a*. *H,* Mass of entangled vegetative cells at the edge of a particle of potato tissue; growth appears to channel into the tissue (at right); silage sample 42 days old.

The spore counts reveal that active spore formation took place during the fermentation, which would suggest that the principal organisms involved were sporulating types. This view gains further support from the fact that all principal colonies isolated from routine plates proved to be Gram-positive spore formers. This is not surprising in view of the degree of heat resistance required by any group surviving both the steaming operation and the period of elevated temperature of the mass during the first 12 days in the silo. The counts further showed that the individual spores fell into two general classes, those that were spherical, and those that were oval (fig. 3, *F*). There seemed to be at least two distinct sizes of oval spores. The majority of the spores counted were free from the cells.

CLASSIFICATION OF THE PREDOMINATING GROUP

During the active fermentation period, a number of isolates representative of each predominating colonial type were made from the dextrose tryptone agar and nutritive caseinate agar routine plates. Thus far sufficient work has not been done to assure identification other than classification within the genus *Bacillus* (Cohn) as thermophiles in group X, as listed by Bergey et al. (*1*). Furthermore, they are nongas-producing, spore-bearing, acid-producing rods, which are facultative with respect to oxygen and temperature requirements. One culture in particular is of interest in that it does not appear to ferment dextrose broth or give visible growth on dextrose or plain agar slants at either 35° or 55° C. However, excellent growth and acid production are obtained in liver broth and in liver agar stabs at both of these temperatures.

In general, liver broth and liver agar were more satisfactory than other media for the cultivation of the cultures isolated from the fermentation. Also, during the latter part of the routine platings, plain liver agar was used in conjunction with the routine media for 55° and 35° C. counts with superior results. With the few trials that were made, liver agar plus bromcresol purple gave higher total counts, larger colonies, and faster growth than the routine media used in this study.

QUALITY OF THE SILAGE

At each sampling period the potatoes were examined to determine the general silage quality. The core samples were all judged good and had a characteristic aromatic odor, and acid taste, and firm texture. The texture resembled that of a boiled potato when cold. The ensiled material formed a more or less solid mass of potatoes pressed tightly together and was free from channels and gas pockets (fig. 1, *G* and *H*). It was not mushy, and no free liquid was present. The aromatic nature of the silage appeared to become stronger with the age of the sample and suggested the presence of a mixture of alcohols, organic acids, and esters. Furthermore, the older samples gave some indication of starch hydrolysis or other structural changes not present in those taken earlier. There was no appreciable color change of the potatoes resulting from the fermentation. The surface layer of potatoes, directly underneath the paper covering was considered spoiled since it had an unpleasant odor and soft texture.

SUMMARY AND CONCLUSIONS

The results of a bacteriological examination of the fermentation of hot-ensiled steamed potatoes (*Solanum tuberosum*) have been presented. The bacteriological findings indicated that the thermophilic, facultative anaerobes were the predominating micro-organisms during the fermentation and were responsible for the developed acidity and resultant preservation of the silage. They are considered to be non-gas-producing, acid-forming, spore-bearing rods, which are facultative with respect to oxygen and temperature requirements. These organisms may be classified according to Bergey et al. as thermophiles belonging in group X of the genus *Bacillus*.

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A CYTOGENETIC STUDY DEALING WITH THE TRANSFER OF GENES FROM TRITICUM TIMOPHEEVI TO COMMON WHEAT BY BACKCROSSING [1]

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INTRODUCTION

Correlated genetic and cytological studies have only infrequently accompanied the transfer of desirable genes from one species to another by means of repeated backcrossing. This paper describes such a study of the hybrid between *Triticum vulgare* [3] Vill. ($n=21$) and *Triticum timopheevi* Zhuk. ($n=14$).

REVIEW OF LITERATURE

Triticum timopheevi, a species endemic to the foothills of the Caucasian mountains, was first described as a variety of *T. dicoccum* Schrank. var. *dicoccoides* Körn. by Zhukovskii *(37)*[4] in 1923. In 1928 it was elevated to specific rank under the name *T. timopheevi* Zhuk. *(38)*. Zhukovskii *(38)* and Sando *(29)* have given detailed botanical descriptions of the species.

The new species soon attracted considerable attention, not only because of its high disease resistance *(5, 13, 17, 26, 28, 31)* but also because of the report by Kihara and Lilienfeld *(19)* that it possessed a genome, designated GG, not previously known to occur in the wheat genus. Kostoff *(20)*, however, regarded the second genome of *Triticum timopheevi* as only a modification of the BB genome of the emmer wheats and designated it $\beta\beta$. Love *(24)* preferred to assign no distinctive formula for the species, stating that it differed from the other 28-chromosome wheats in degree of divergence only. Svetozarova *(33)* has since shown that 14 bivalents occur in the hybrid of *T. timopheevi* with *T. armeniacum* (Jacubz.) Makush. ($n=14$); presumably these species are more closely related to each other than to other 28-chromosome wheats.

Triticum timopheevi has been demonstrated to be highly sterile in crosses with all other species of wheat including *T. armeniacum (17,*

[3] The priority of *Triticum aestivum* L. for the name of the species more commonly known as *T. vulgare* is recognized. Because the latter name is in general use by agronomists the world over, preference is given to this form.

[4] Italic numbers in parentheses refer to Literature Cited, p. 62.

19, 20, 28, 31, 33).   Despite this sterility some of its disease resistance was transferred to common wheat by Pridham (*28*).   He was able to select fertile lines resistant to stem and leaf rusts and of fair agronomic type by the F_5 generation.   Shands (*31*) obtained fertile stem- and leaf-rust-resistant lines with 21 pairs of chromosomes following one backcross to *T. vulgare* (the male parent) and 5 generations of selfing.

A general review of the cytology of interspecific wheat hybrids has been given by Aase (*1*).   Worthy of special mention was the demonstration by Kihara (*18*) that the parental chromosome numbers were regained during several generations of self-pollination of the moderately fertile pentaploid wheat hybrids.   According to Kihara, two series were formed, an ascending series from which 42-chromosome plants resulted eventually, and a descending series which resulted in 28-chromosome plants.   The ascending series was characterized by 15 to 21 pairs of chromosomes with the sum of the bivalents and univalents numerically equal to 21.   The descending series was characterized by 14 pairs plus 1 to 6 univalents, the univalents gradually being lost until a condition of 14 pairs was reached.   *Vulgare*-like plants (ascending series) were much less frequent than emmer-like plants (descending series); this observation has since been voluminously confirmed (*11, 12*).   Certain *Nicotiana* hybrids also conform to Kihara's scheme, as shown by Lammerts (*21*).   Despite the return to parental chromosome numbers meiotic instability may persist for many generations in derivatives from pentaploid wheat hybrids (*23, 25, 30*).

In interspecific hybrids a greater proportion of female gametes than male gametes are usually functional (*14, 21, 34, 36*), although exceptions occur, as in certain *Gossypium* hybrids (*8, 9, 10*).   When pentaploid wheat hybrids are backcrossed to the common wheat parent, there is severe selection against male gametes with intermediate chromosome numbers, but female gametes with 14 to 21 chromosomes function about equally frequently (*34, 36*).   Thus, in plant hybrids, a striking difference frequently has been noted in the progenies derived from reciprocal backcrosses.

The backcross method has been used by several investigators to increase fruitfulness in the progenies of interspecific hybrids.   Three or four backcrosses have usually been sufficient to restore apparently complete fertility in several highly sterile hybrids (*4, 8, 9, 10, 14, 15, 32*).

There is rather general agreement concerning the number of backcrosses necessary to recover the type of the recurrent parent.   Harland and Atteck (*10*), working with interspecific *Gossypium* hybrids, reported that "generally speaking from the results of all the backcrossing experiments, it appears to be unnecessary to carry the backcrossing beyond the fourth or at most the fifth backcross, as the heterozygote is by then stabilized on the new genetical background."   Holmes (*16*) reached essentially the same conclusion in work with interspecific tobacco hybrids.   The most extensive investigations of intraspecific backcrosses are those of Briggs (*3*), whose work with small grains indicated that after three or four successive backcrosses the derivative populations had become so nearly like the recurrent parent that selection for characters other than the one being trans-

ferred was not effective. Six backcrosses were regarded as necessary, however, to recover the recurrent parent in close detail.

MATERIAL AND METHODS

The strain of *Triticum timopheevi* used in this investigation was brought to the United States by Dickson [5] as P. I. [6] 94761. An unnamed spring wheat selection out of the hybrid Illinois No. 1 × Chinese, designated as 2666A2–2–15–6–3 by the Wisconsin Agricultural Experiment Station, was used as the common wheat parent because it was known to function better than several other varieties in hybrids with *T. timopheevi*. The parents will hereafter be designated *vulgare* and *timopheevi*.

In making crosses, spikelets at the two or three top and bottom nodes of the spike, and all but the first two florets of the remaining spikelets, were removed. The remaining florets were emasculated, protected with a glassine bag, and pollinated 1 to 3 days later.

Seed set was determined by calculating the percentage of pollinated florets or first and second florets of central spikelets on open-pollinated or selfed spikes that produced seeds. Self-pollination was enforced by covering some spikes with glassine bags from 2 or 3 days before anthesis to maturity. Backcross fertility was calculated from not less than 40 florets and self-fertility or open-pollinated fertility from not less than 100 florets per plant.

Using V and T to represent *vulgare* and *timopheevi*, respectively, the following will outline the symbols used to designate hybrids, backcrosses, and the selfed generations therefrom. The seed parent is written first and the pollen parent second throughout.

Origin: *Designation*
 $V \times T$ -- F_1.
 F_1 hybrid allowed to open-pollinate -------------------- F_2 (o. p.).[7]
 F_1 hybrid protected from outcrossing -------------------- F_2.
 $V \times (V \times T)$ -- V(VT).
 $(V \times T) \times T$ -- (VT)T.
 $(V \times T) \times V$ -- BC^1.
 $[(V \times T) \times V] \times V$ -------------------------------- BC^2.
 $V \times [(V \times T) \times V]$ -------------------------------- RBC^2.[8]
 $V \times (V \times [(V \times T) \times V])$ -------------------------- RBC^3.
 BC^1 protected from outcrossing ---------------------- BC^1F_2.
 BC^1 allowed to open-pollinate ---------------------- BC^1F_2 (o. p.).
 Unless indicated by (o. p.), pollination was controlled in all cases.

All cytological observations were made from acetocarmine smear preparations. Twenty-five pollen mother cells (P. M. C.) at metaphase of the first meiotic division were analyzed for pairing from each of the parental plants, from the F_1, from V (VT) plants, and from 40 BC^1 plants. In the remaining BC^1 plants and in all other generations, only 10 P. M. C. per plant were analyzed. One-hundred microspores still in the form of quartets were examined in each plant to determine the

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[5] DICKSON, J. G. CEREAL DISEASES STUDIES IN EUROPE AND ASIA. 1930. [Unpublished manuscript in the University of Wisconsin library.]

[6] P. I. refers to the accession number of the Division of Plant Exploration and Introduction, United States Department of Agriculture (formerly Foreign Plant Introduction).

[7] (o. p.) stands for open-pollinated.

[8] R stands for reciprocal. Superscript 2 is used to emphasize that this generation received the same number of backcrosses to *vulgare* as BC^2, although only one of the two backcrosses was in the reciprocal direction

frequency of micronuclei. Microcytes were tabulated separately, but because of their sporadic occurrence were later included as micronuclei.

The amount of good pollen was determined from anthers collected at anthesis, fixed in Carnoy's solution, and stained with acetocarmine. Only those pollen grains were considered "good" which possessed two gametes and a vegetative nucleus, the condition characteristic of the pollen of both parents. Estimates of the percentage of good pollen were based upon the examination of 200 pollen grains per plant.

In order to obtain an unbiased sample of pollen mother cells, quartets of microspores, or pollen grains, all units were counted within random strips from one side of the slide to the other.

An attempt was made to obtain a random sample from each back-cross generation for morphological and cytological study by determining by random methods before planting the plants to be studied. Not less than 20 hybrid plants were used as parents to provide as wide a base as possible for each backcross generation. Many families in excess of those studied cytologically or morphologically were grown in separate nurseries, and were useful for studies of disease resistance and for additional observations upon morphological characteristics.

THE PARENTAL SPECIES

Some contrasting characters of the common wheat parent and *timopheevi* are given in table 1. Spikes are illustrated in figure 1, *A* and *E*. It will subsequently be shown that both parents are fertile and cytologically regular.

TABLE 1.—*Expressions of certain morphological characters observed in vulgare, timopheevi, and in the F_1 hybrid*

Character	Expression of—		
	Vulgare	Timopheevi	F_1 hybrid
Diseases:			
Stem rust	Susceptible	Resistant	Nearly like *timopheevi*.
Bunt	Susceptible	do	Susceptible.
Mildew	Moderately suscep-tible.	do	
Leaf rust	Fairly resistant	do	Moderately susceptible.
Glume characters:			
Adherence to kernel	Easily detached	Attached tightly	Like *timopheevi*.
Keel	Inconspicuous	Prominent	Do.
Shoulder	Nearly wanting	Narrow with pointed tooth.	Do.
Tip	Beak 5 to 10 milli-meters, increasing toward apex of spike.	Beak 5 millimeters or less in length.	Do.
Pubescence	Absent	Long, dense	Intermediate in length and density.
Other characters:			
Lemma	Awned	Awned	Awned.
Rachis articulation	Tough	Moderately fragile	Like *timopheevi*.
Adherence of spikelet to rachis.	Easily detached	Tenacious	Do.
Leaf pubescence	Absent	Long, dense	Intermediate in length and density.
Ciliation of leaf sheath margin.	do	do	Do.
Spike density [1]	5.3 millimeters	2.9 millimeters	4.4 millimeters.
Maturity	Midseason	Late	Very late.
Kernel type	Ovate, red	Long, slender, light red.	Very short, intermediate red.
Vigor	Vigorous	Vigorous	Very vigorous.
Straw	Thick	Slender	Intermediate.

[1] Mean length of the 10 central internodes of the spike.

FIGURE 1.—Spikes: *A, vulgare* parent; *B*, V (VT); *C, vulgare* × *timopheevi* F₁; *D, timopheevi* ×*vulgare* F₁ ; *E, timopheevi* parent.

THE F₁ GENERATION

Of 375 florets of *vulgare* that were pollinated with pollen of *timopheevi*, 75 percent set seed.   This was comparable to the success of intervarietal crosses in *vulgare* made at the same time.   The F₁ kernels

were shorter and smaller than selfed kernels of *vulgare*, but had plump endosperms; 89 percent germinated. The reciprocal cross gave a slightly higher seed set. These kernels were badly shrunken, however, and only 4 of 79 germinated. The F_1 plants of the reciprocal crosses were indistinguishable in appearance (fig. 1, *C* and *D*), cytological behavior, and fertility.

The F_1 hybrids were similar to *timopheevi* in some characters, but in most others they were intermediate between the parents (table 1). Except for reaction to bunt, no character of the *vulgare* parent appeared to be governed by dominant genes. The F_1 hybrids were large, sturdy, and tillered profusely.

The 11 F_1 plants examined cytologically each had 35 chromosomes (fig. 2, *C*). Microsporogenesis was similar to that observed by Kostoff (20), Love (24), and Pathak (27), and was in sharp contrast to the regular meiotic behavior of the parents (fig. 2, *A* and *B*, and table 2). The variability in pairing is readily noted from the range in the numbers of each type of association of chromosomes and also by the 42 different combinations of univalents, bivalents, trivalents, and multiple associations. When open and closed [9] bivalents were considered to be different types of association, the numbers of associations exceeded 250 in the 275 pollen mother cells examined.

TABLE 2.—*Meiotic behavior of the parental species, the F_1 hybrid, and of the back-cross with the F_1 as the pollen parent* [1]

Cytological character	Vulgare	Timopheevi	F₁ hybrid	V(VT)
Number of plants examined cytologically	11	8	11	11
2n chromosome number	42	28	35	35
Bivalents:				
Average	20. 86	14. 00	8 29	9 03
Range	19–21	0	4–13	5–14
Closed bivalents:				
Average	19. 32	12 74	3 22	3. 69
Range	14–21	10–14	0–7	1–7
Open bivalents:				
Average	1 54	1. 26	5. 07	5 34
Range	0–7	0–4	1–9	1–9
Univalents:				
Average	0 16	0	14.79	13 89
Range	0–4	0	7–21	7–21
Trivalents:				
Average	0. 01	0	1. 04	0. 89
Range	0–1	0	0–4	0–4
Quadrivalents:				
Average	0. 01	0	0 13	0. 13
Range	0–1	0	0–1	0–1
Quinquevalents:				
Average	0	0	0. 01	0. 01
Range	0	0	0–1	0–1
Number of different pairing arrangements	4	1	42	40
Number of micronuclei per quartet:				
Average	0 15	0. 04	4. 98	4. 89
Range	0–5	0–5	0–12	0–13
Percent quartets with no micronuclei	92	98	1. 3	1. 0
Percent good pollen	84	94	1. 7	0. 8
Seed set:				
Open-pollinated	94	97	0. 6	0. 1
Backcross			5. 35	6. 52

[1] See Materials and Methods for the number of observations made per individual plant.

[9] Open bivalents have one or more chiasmata confined to a single arm, abbreviated IIo hereafter. Closed bivalents have one or more chiasmata in each arm, giving closed configurations, abbreviated IIc. Univalents and trivalents are abbreviated I and III respectively.

Observations on serial sections of four megaspore mother cells indicated that the chromosome behavior was similar in both megasporogenesis and microsporogenesis. This agrees with the results of Watkins (35) based upon extensive studies of the hybrid *Triticum vulgare* × *T. turgidum*.
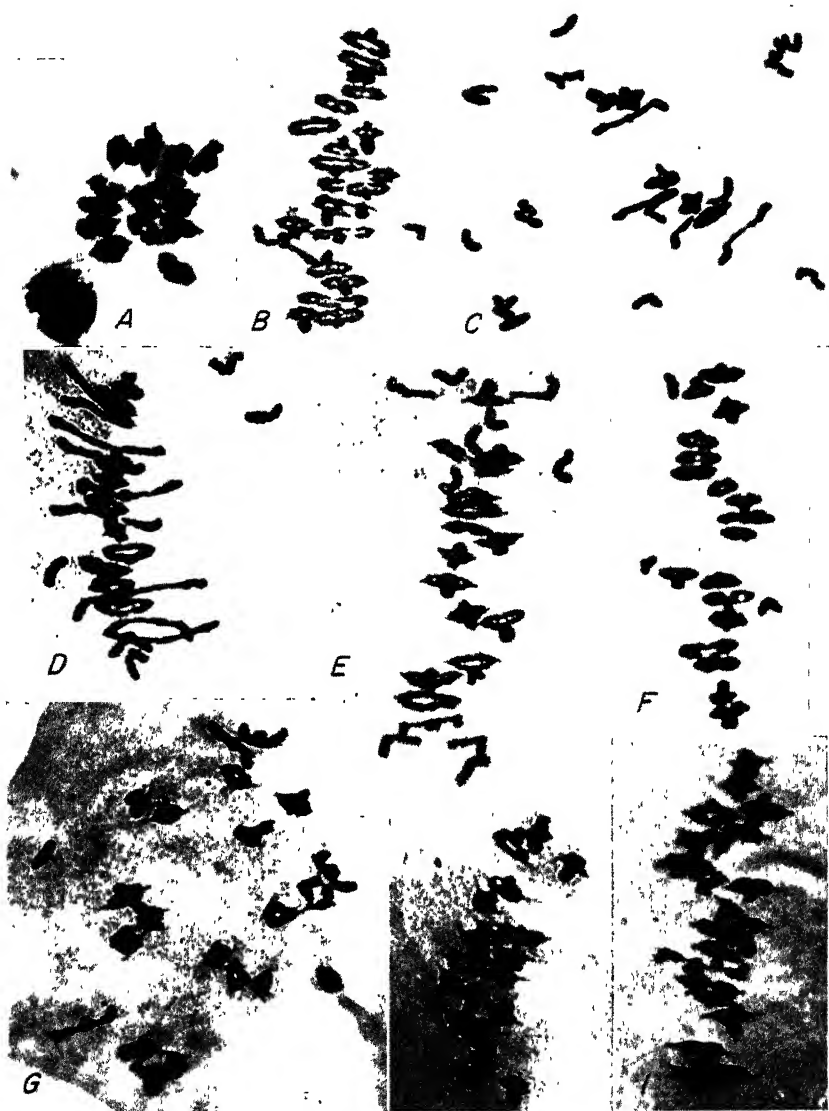
FIGURE 2.—First meiotic metaphases: *A*, 14 IIc in *timopheevi*; *B*, 19 IIc and 2 IIo in *vulgare*, *C*, F₁ hybrid showing 5 IIc, 3 IIo, 16 I, and 1 III; *D*, BC¹ P. M. C. with 10 IIc, 5 IIo, 5 I, and 2 III; *E*, BC² P. M. C. with 14 IIc, 3 IIo, and 6 I; *F*, BC³ P. M. C. with 18 IIc and 3 I; *G*, BC⁴ P. M. C. with 17 IIc, 4 IIo, and 1 I; *H*, RBC³ P. M. C. with 21 IIc; *I*, BC²F₄ P. M. C. with 20 IIc and 1 IIo

Variability in pairing in the hybrid, known autosyndesis in *vulgare*, and lack of recognizable individuality of chromosomes complicate the estimation of homology between the chromosomes of the two species. The occurrence of an average of 3 closed pairs suggests that some chromosomes may be almost completely homologous. Since up to 13 (open and closed) pairs were observed, and if autosyndesis was not occurring, it might be assumed that nearly all of the chromosomes of *timopheevi* have segments homologous to portions of *vulgare* chromosomes.

Incomplete pairing of chromosomes in species hybrids may be determined by chromosome homology, environment, or possibly by genes. An analysis of meiotic behavior in F_1 hybrid plants revealed no significant
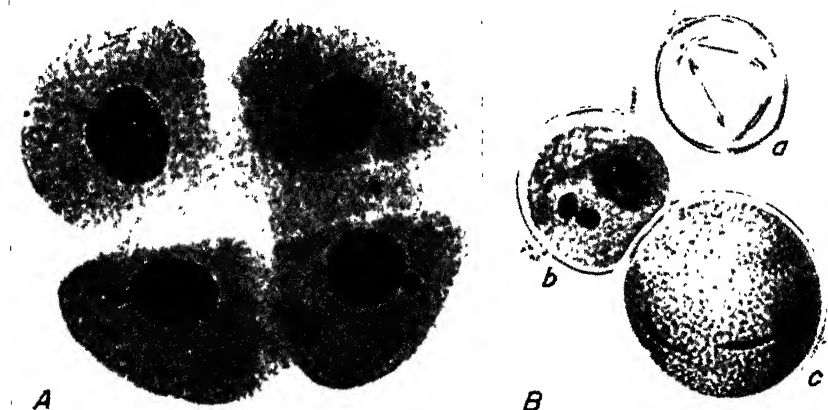
FIGURE 3.—*A*, Quartet of microspores with a single micronucleus in each of two microspores. *B*, Pollen grains: *a*, Aborted and, *b*, retarded pollen grains; *c*, a "good" pollen grain with two male gametes and a vegetative nucleus.

differences in pairing between material collected at 11 different dates from plants grown in both the greenhouse and the field at Madison, Wis., in the period 1941 to 1943. This study has been reported in detail elsewhere.[10] Because the 11 dates included a wide range of conditions, it was concluded that environmental influences did not contribute significantly to the differences in meiotic behavior observed. Since there is also no evidence that gene action is responsible for the incomplete pairing either in the F_1 or later generations, incomplete homology of chromosomes appears the most reasonable explanation. If chromosomes of *timopheevi* varied in their homologies with chromosomes of *vulgare*, it would be expected that genes in the more homologous chromosomes would be easier to transfer to common wheat.

Irregular pairing at meiosis resulted in large numbers of laggards which frequently remained in the cytoplasm as micronuclei (table 2, fig. 3, *A*).

About 1 percent of "good" pollen was found in anthers of F_1 hybrids (table 2, fig. 3, *B*, *c*). The remainder of the pollen was mostly com-

[10] ALLARD, R. W.   A CYTOGENETIC STUDY OF THE EFFECT OF BACKCROSSING TO COMMON WHEAT IN A HYBRID BETWEEN TRITICUM VULGARE VILL. AND TRITICUM TIMOPHEEVI ZHUK.   1946. [Unpublished Ph. D. thesis. Copy on file University of Wisconsin library, Madison.]

pletely empty (fig. 3, *B, a*). A small percentage of grains had some cytoplasm but only one or sometimes two or three nuclei (fig. 3, *B, b*).

A seed set of 5.4 percent was obtained when pollen of *vulgare* was used on the F_1. Under open pollination approximately 0.6 percent of the florets set seed. When isolated in the greenhouse or protected from outcrosses in the field with glassine bags only 5 kernels were obtained from more than 10,000 florets, a seed set of less than 0.05 percent. Plants obtained from the kernels above are discussed under BC_1F_2 (o. p.), and F_2 generations, respectively. Kernels obtained from the backcross V(VT) are considered in the following section.

THE BACKCROSS V(VT)

The backcrosses were made in a greenhouse in which no *timopheevi* was growing, excluding the possibility that the seeds resulted from accidental crossing with that species.

The pollination was performed by breaking open the leathery anthers of the F_1 and brushing them upon the stigmas of *vulgare*. This laborious process resulted in 32 kernels from 1,370 florets. Three of these kernels resembled *vulgare* and produced plants of that type, probably the result of accidental selfing or outcrossing. The remote possibility exists, however, that pollen grains from F_1 plants which had exactly the monoploid complement of *vulgare* effected pollination. Twenty-nine of the 32 kernels resembled F_1 kernels in appearance. Of these, 25 germinated and produced mature plants which were morphologically indistinguishable from each other and from plants of the F_1 generation (fig. 1, *B*).

Eleven of the 25 backcrossed plants were examined cytologically and all were found to have 35 chromosomes (table 2). Conjugation was similar to that in F_1 plants and they were also similar in frequency of micronuclei, proportion of good pollen, and fertility (table 2). However, there was a significantly greater number of pairs and fewer univalents in the backcrossed population than in the F_1 population.[11] This provides evidence that crossing-over had occurred between some chromosomes and that functional pollen of the F_1 on the average possessed slightly more chromatin homologous to that of common wheat than did pollen of *timopheevi*. Alteration of chromosomes carried by functional F_1 pollen was slight, however, and backcrosses in this direction were discontinued when it became evident that only slow progress in transferring genes of *timopheevi* to common wheat could be expected.

THE BC¹ GENERATION AND ITS DERIVATIVES

MORPHOLOGICAL REGRESSION TOWARD THE RECURRENT PARENT

The pollination of 5,425 flowers of F_1 plants with pollen of *vulgare* resulted in 290 kernels, of which 119 germinated and produced mature plants. In contrast to the backcross V(VT), a wide range of segregation occurred, all plants, however, varying about the *vulgare* type (fig. 4, *B–D*). With further backcrosses to *vulgare* the population assumed an even more *vulgare*-like appearance (fig. 5, *B–D*). A random group of several hundred plants from backcross and backcross derivative generations was classified for the morphological characters

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[11] See footnote 10, p. 40.

listed in table 1. The plants were divided into 5 classes varying from like *vulgare* to like *timopheevi*. A detailed account of this classification has been presented elsewhere.[12]
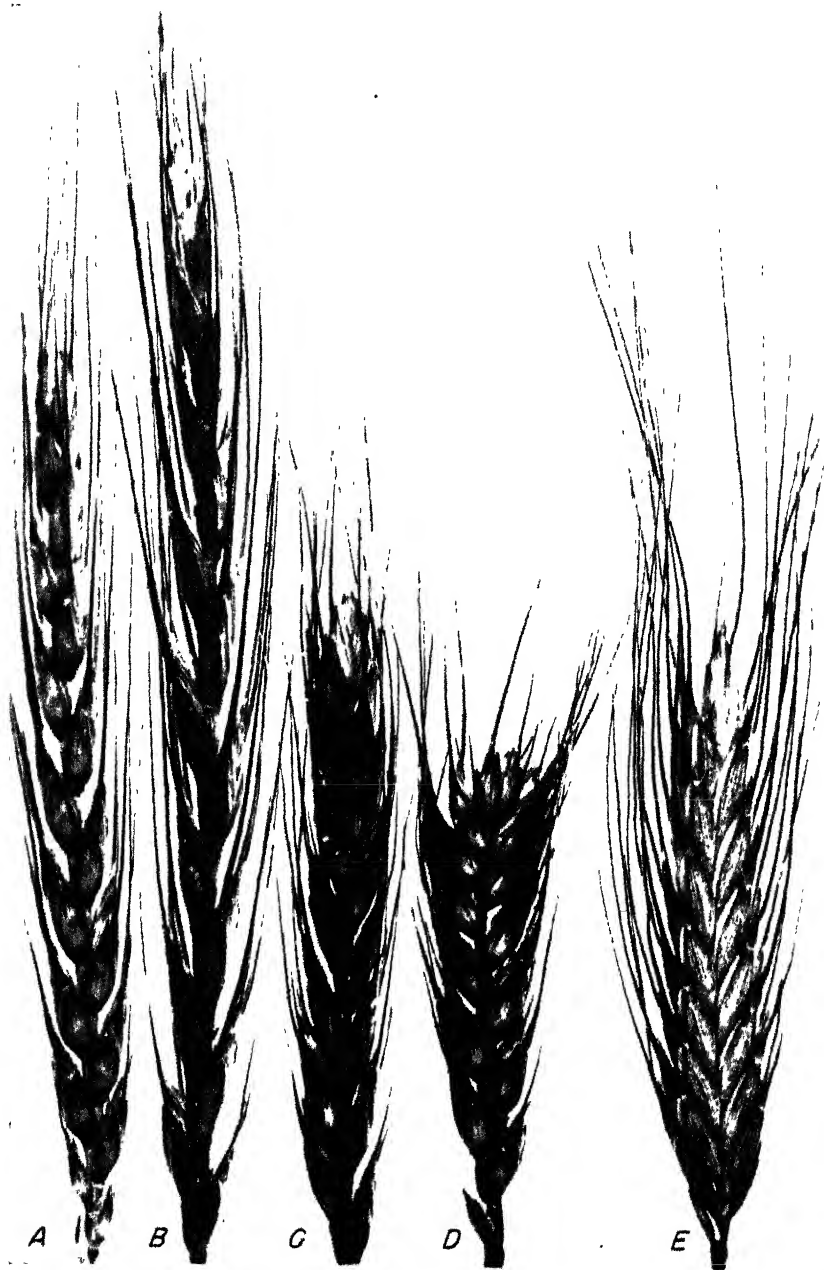
FIGURE 4.—Spikes: *A, vulgare; B–D*, some spike types observed in the BC[1] generation; *E, timopheevi*.
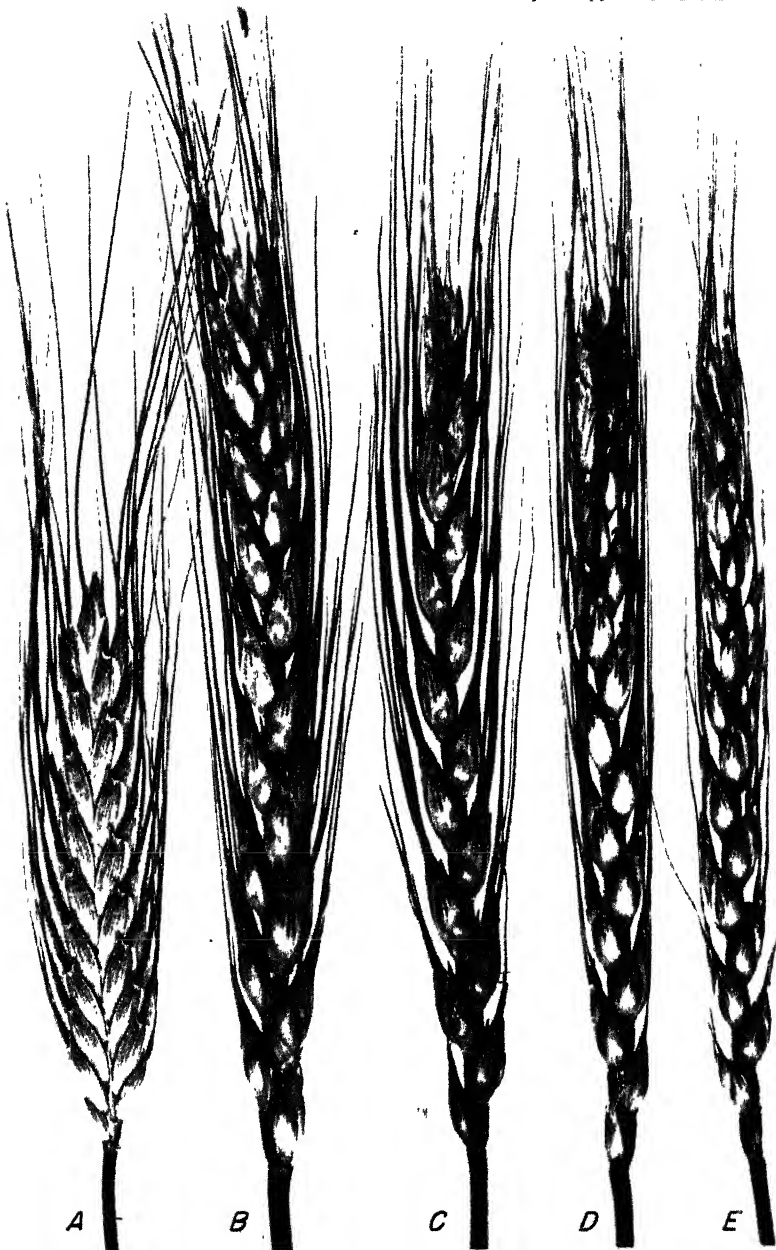
FIGURE 5.—Spikes: *A, vulgare; B–D*, some spike types observed in the BC⁴ generation; *E, timopheevi*.

The results indicated that different characters disappeared from the population or lost their *timopheevi*-like expression at different rates with successive backcrosses. For example, the *timopheevi*-like shape of the glume beak, the keel on the glume, and the glume shoulder

appeared to be retained longer than other characters studied. Though all 3 are glume characters, they appeared independently in different plants and hence must be governed by different genes. More than 90 percent of the BC[1] plants resembled *timopheevi* more closely than *vulgare* in these characters. Even after 3 backcrosses the glume keels of 54 percent of the population resembled those of *timopheevi;* and the 2 other characters, shape of glume beak and shoulder, were *timopheevi*-like in 44 and 19 percent of the BC[3] population, respectively. After 4 backcrosses many plants retained a *timopheevi*-like expression of these characters. The 3 glume characters occasionally became fixed in *vulgare*-like lines by selfing backcrossed plants.

A group of characters somewhat less strongly retained in backcrossed populations were pubescence on the laminae, glumes, and cilias on the margins of the leaf sheaths. These characters appeared independently in different plants. Although 38 to 52 percent of the BC[1] plants possessed pubescence of intermediate length and density, three backcrosses served to eliminate pubescence almost completely. Pubescence became fixed in some lines following selfing, but much less frequently than the glume characters discussed earlier.

Unlike the six previously mentioned characters which are economically unimportant, the tight envelopment of the caryopsis by the glumes, lemma, and palea, the strong adherence of the spikelet to the rachis, and the easy articulation of the rachis are characters of *timopheevi* which are very undesirable economically because they contribute to preharvest shattering and threshing difficulties. These characters appeared in nearly all BC[1] plants but usually with an expression intermediate between the parental species. After three backcrosses few if any traces of difficult threshing remained, and four backcrosses eliminated these characteristics completely. Rarely did these characters become fixed in lines derived by selfing early backcross generations.

Classification of plants of backcrossed populations for kernel type, maturity, leaf and straw type, and spike characters proved difficult because of numerous intergrades. However, the *timopheevi*-like expression of these characters usually failed to survive more than one or rarely two backcrosses to *vulgare*.

A striking difference in the rate of regression toward the recurrent parent was noted between the BC[2] and RBC[2] populations. When BC[1] plants were the pollen parents almost all *timopheevi* characters were screened out so that the RBC[2] population much more closely resembled the recurrent parent than when BC[1] plants were the seed parents. The RBC[3] population also more closely resembled the recurrent parent than the comparable BC[3] population. Thus, the functional male gametes of BC[1] plants were genetically much more like *vulgare* than were the functional female gametes.

In summary, it may be said that a single backcross to *vulgare* as the male parent resulted in a highly diverse population which resembled that species grossly, but retained many characters of *timopheevi* in a form at least intermediate between the parents. During two more backcrosses the characters of *timopheevi* were lost or diluted, some more rapidly and completely than others, and the backcross population became much more uniform. However, after four back-

[12] See footnote 10, p. 40.

crosses the population still varied rather widely about the type of the recurrent parent even though few evidences of *timopheevi* parentage remained. The uniformity expected on the basis of published accounts (*9, 10, 15*) was lacking, although a few individuals nearly identical with the recurrent parent were found. Backcrosses with BC[1] and RBC[2] plants as pollen parents resulted, however, in a much more rapid return to the type of *vulgare*. Studies of the transmission of disease reaction, which is economically more important than the characters discussed above, are reported in the two following sections.

RUST STUDIES

The reaction of several generations to stem rust caused by *Puccinia graminis tritici* Eriks. and Henn. was determined in the field in the summers of 1942, 1943, and 1944. Epiphytotics of the disease were induced by inoculation early in the season of guard rows of a susceptible variety with races 19 and 56. Other races were known to have occurred naturally. The most important of these races was 15B, which appeared in the nursery in 1943. Race 15B was identified by Dr. E. C. Stakman, of the University of Minnesota, who also furnished the inoculum of races 19 and 56. Variety 2666A2–2–15–6–3, the *vulgare* parent, proved quite resistant to leaf rust caused by *Puccinia triticina* Eriks. and none of the backcrossed progeny showed more than a light infection by this disease in any of the several years they were under observation. Some of the backcrossed plants and their derivatives possessed a very high order of resistance to leaf rust that apparently came from the *timopheevi* parent.

The "host response" to stem or leaf rust was determined by comparison with the standard scale for estimating rust precentages adopted by the Division of Cereal Crops and Diseases, United States Department of Agriculture (*22*). The severity of infection expressed as a percentage figure was also recorded for each plant. Both scales were later used in determining degree of resistance.

In each season a satisfactory epiphytotic of stem rust developed. Susceptible varieties such as the *vulgare* parent became infected early in the season. Soon after heading, the severity of infection reached 90 percent on most plants with pustules of the very or completely susceptible type, and only shriveled caryopses were produced. *Timopheevi* had an apparently immune type of "host response" with pustules appearing only rarely and then late in the season. In 1943 occasional plants of *timopheevi* had up to 1 percent of rust, possibly because of the presence of race 15B. The F_1 appeared to be highly resistant until heading time, when a few pustules were observed. These increased until 5 percent severity of infection was reached near maturity in 1942 and 1944 and 10 percent in 1943. The stem rust resistance of *timopheevi* is thus not completely dominant to the susceptibility of the *vulgare* parent.

In the 1943 season 70 BC[1] plants were tested for stem rust reaction. They appeared to fall into three groups on the basis of host response and severity of infection: (1) Plants which were entirely free from rust or developed a few pustules of the apparently immune type late in the season; (2) plants with a fairly resistant host response and of variable severity of infection, mainly from 1 to 5 percent, but occasionally reaching 40 percent in late-maturing plants; and (3) plants as suscep-

tible as the common wheat parent. The 70 BC^1 plants fell into the resistant, intermediate, and susceptible groups in the proportion of 28:31:11, respectively. Simultaneously 169 BC^2 individuals from 22 different BC^1 plants and 43 RBC^2 individuals from 7 BC^1 plants were tested for stem rust reaction. The backcrosses had been made in the greenhouse so that the rust reaction of the BC^1 parents was unknown. The proportion of resistant, intermediate, and susceptible plants was 43:72:54 for the BC^2 and 20:11:12 for the RBC^2 progenies. Five additional BC^2 families with a total of 52 individuals included only susceptible plants. Genes for rust resistance were thus transmitted through the pollen as frequently as through the eggs of hybrid plants.

The ratios between resistant, intermediate, and susceptible plants in the BC^1, BC^2, and RBC^2 generations, together with the appearance of numerous completely susceptible plants, suggested that the inheritance of *timopheevi* to stem rust might be simple, perhaps depending on not more than two major genes. To test this hypothesis, backcrossed progeny and some selfed progeny from single plants of known rust reaction were grown in 1944.

Only susceptible individuals were found in 438 progeny of susceptible plants of the BC^3, BC^2F_2, and BC^2F_4 generations. Apparently none of the resistance of *timopheevi* to races of rust occurring at Madison in 1944 was governed by recessive genes.

In 1944 almost no plants of the intermediate reaction were found, possibly because race 15B was not prevalent. The races of stem rust occurring in 1944 would not differentiate between the progeny of the intermediate and resistant plants of 1943. For example, 107 BC^3 progeny of 10 different resistant BC^2 plants segregated into resistant, intermediate, and susceptible individuals in the proportion of 56:2:49, respectively. The proportion for BC^3 progeny of 6 different BC^2 plants of intermediate resistance was 34:3:34. Both backcross ratios suggest that a single major gene governs resistance, if the somewhat higher incidence of rust on intermediate plants resulted from local environmental effects rather than genetic differences.

A large number of F_2 or F_3 segregates from BC^2 or RBC^2 and F_4 segregates from BC^1 plants were tested in 1944. The first would be expected to give F_2 ratios and in the F_3 and F_4 populations both segregating and homozygous lines could occur. In segregating lines the proportion of resistant : intermediate : susceptible plants was 1,952: 225:1,203. Although all attempts at a factorial analysis were unsuccessful, the proportions illustrate the ease with which stem-rust resistance was maintained.

Of the 11 BC^1F_4 families grown, 4 families of 11, 27, 45, and 89 individuals included only resistant plants, indicating that their BC^1F_3 parents were homozygous for resistance to stem rust. Several of these lines have been grown subsequently into the F_5 to F_7 generations and have been uniform for a high type of resistance, establishing the fact that homozygosity for resistance to stem rust occurred in F_3 individuals.

Although the data did not establish the number of genes governing the inheritance of *timopheevi* resistance to stem rust, they do suggest that relatively few major genes, possibly not more than 2, control the resistance to races which occurred at Madison in 1943 and 1944. At any rate a high order of resistance could be fixed by the F_3 generation

(from the first backcross), and a large proportion of resistant plants occurred in random populations after 4 backcrosses to *vulgare*. To determine the genic basis of resistance it is probable that studies of cytologically stable, 42-chromosome, homozygous resistant lines, crossed with other common wheats and tested with single races of rust will be necessary.

<div align="center">BUNT STUDIES</div>

Studies of transmission of resistance to bunt were made in the greenhouse in 1943–44 and in 1944–45. A composite collection of bunt from three localities in Wisconsin was used as inoculum. All were smooth-spored types of the species *Tilletia foetida* (Wallr.) Liro. Seeds were dusted with chlamydospores of the causal organism and planted in soil maintained at approximately 10° C. The seeds were then covered with sand mixed with chlamydospores.

In the 1943–44 tests four groups of kernels were inoculated: F_1, BC^2, BC^1F_2 (o. p.), and RBC^2F_2 (o. p.) kernels. Progress and Reward, highly susceptible varieties, and the *vulgare* parent were grown as checks. More than 90 percent of the plants of these varieties were infected and showed the typical symptoms of the bunt disease. The F_1 plants were susceptible, but the symptoms were less pronounced than in the susceptible varieties. The plants were only slightly stunted if at all and very small spore balls were formed. Usually the disease did not involve the entire spike, but only a few spikelets.

BC^2 families varied widely in the proportion of bunt-infected plants; on the average they had slightly less disease than the *vulgare* parent. In 3 of 19 families tested, more than 50 percent of the individuals remained healthy, ratios of healthy to diseased plants in those families being 11:9, 4:1, and 10:9. The BC^1F_2 (o. p.) plants were considerably more resistant on the average than was the *vulgare* parent. In 10 of 15 families more than one-half of the plants remained healthy. In one family 20 of 23 and in another, 17 of 26 plants remained healthy. There was no indication that any of 18 RBC^2F_2 (o. p.) families possessed any more resistance to the disease than the common wheat parent, although their BC^1 parents apparently transmitted resistance to certain BC^2 and BC^1F_2 families. This suggests that resistance to bunt is less frequently transmitted by pollen than by eggs of segregating plants.

In 1944–45 tests all kernels obtained from bunt-free plants in 1943–44 were grown. Again, a few families had more healthy than bunted plants. Likewise, numerous partly bunted plants, which seemed to possess some measure of resistance to the disease, were found.

The foregoing results suggest that it may be possible to transfer some of the bunt resistance of *timopheevi* to *vulgare* types.

REGRESSION TOWARD THE RECURRENT PARENT IN CYTOLOGY AND FERTILITY

All chromosome numbers from 36 to 42 were observed in BC^1 plants (table 3). Because the male gametes from *vulgare* presumably had 21 chromosomes, functional female gametes of F_1 plants apparently possessed 15 to 21 chromosomes. Three additional backcrosses served to shift the mean chromosome number from 39.1 in the BC^1 generation to 41.6 chromosomes in the BC^4 population (table 3).

TABLE 3.—*Distribution of wheat plants of several generations according to meiotic behavior, frequency of micronuclei, proportion of good pollen, and fertility*

[The mean represents the mean value for each generation, while the range represents the maximum and minimum values for single plants within each generation]

Cytological character and seed set	Generation										
	Vulgare	BC¹	BC²	BC³	BC⁴	RBC²	RBC³	BC¹F₂	BC²F₂	F₂ (o. p.)	F₃ (o. p.)
Chromosome number:											
Mean	42.0	39.1	40.6	41.2	41.6	42.1	42.2	41.0	41.8	39.7	41.2
Range	None	36–42	38–44	37–44	41–43	41–43	42–43	35–44	40–43	37–42	40–43
Number of plants	11	75	88	68	19	31	13	22	15	18	12
Closed bivalents:											
Mean	19.3	10.1	13.5	17.1	18.2	16.0	17.9	14.1	16.7	10.2	14.5
Range	18.3–20.1	5.4–14.9	9.0–19.1	12.7–20.0	16.1–20.4	12.7–19.3	16.6–19.0	8.2–18.6	12.1–20.0	8.1–13.3	11.9–16.8
Number of plants	11	75	88	68	19	31	13	22	15	17	11
Open bivalents:											
Mean	1.5	4.4	3.9	2.4	1.8	3.5	2.4	4.3	3.2	4.3	3.5
Range	0.8–2.4	2.6–7.0	0.7–6.9	0.4–1.9	0.6–3.6	1.2–5.6	1.1–3.5	2.2–6.7	1.0–7.0	3.2–6.1	1.1–3.6
Number of plants	11	75	88	68	19	31	13	22	15	17	11
Total bivalents (open and closed):											
Mean	20.9	14.4	17.4	19.5	20.0	19.4	20.3	18.4	19.9	14.5	18.0
Range	20.4–21.0	10.6–18.3	13.9–20.8	17.1–21.0	18.8–21.0	15.2–20.8	19.2–21.0	14.9–20.8	16.8–21.0	12.9–16.8	16.9–20.7
Number of plants	11	75	88	68	19	31	13	22	15	17	11
Univalents:											
Mean	0.2	7.6	4.4	1.7	0.7	2.3	1.1	3.4	1.6	7.8	3.1
Range	0.1–0.2	3.5–13.9	0.2–9.7	0–5.9	0–2.4	0.2–5.5	0–2.9	0.4–5.3	0–5.8	4.3–13.3	1.3–6.1
Number of plants	11	75	88	68	19	31	13	22	15	17	11
Trivalents:											
Mean	0.01	0.7	0.4	0.1	0.2	0.2	0.1	0.3	0.1	0.9	0.7
Range	0–0.8	0–2.1	0–1.6	0–1.5	0–0.9	0.1–1.2	0–0.7	0–0.7	0–0.4	0–1.9	0–1.9
Number of plants	11	75	88	68	19	31	13	22	15	17	11
Micronuclei per quartet:											
Mean	0.2	2.3	1.6	0.9	0.3	0.7	0.7	1.4	1.2	------	------
Range	0.04–0.3	0.7–5.6	0.1–6.5	0.1–2.6	0.03–0.6	0–2.7	0–1.6	0.3–4.6	0.2–4.8	------	------
Number of plants	10	48	72	37	17	23	10	12	8	------	------
Percent quartets with no micronuclei:											
Mean	92	11	33	51	80	58	59	45	50	------	------
Range	91–98	0–75	0–90	5–95	56–97	14–91	21–91	3–95	0–88	------	------
Number of plants	10	48	72	37	17	23	10	12	8	------	------
Percent good pollen:											
Mean	95	23	51	65	------	75	------	69	------	------	------
Range	51–97	1–60	4–95	2–96	------	43–94	------	14–94	------	------	------
Number of plants	58	97	94	37	------	10	------	13	------	------	------
Open-pollinated seed set (percent):											
Mean	94	9	35	56	------	77	------	43	------	------	------
Range	73–96	0–85	0–95	15–95	------	5–95	------	5–91	------	------	------
Number of plants	73	66	197	47	------	28	------	22	------	------	------

Backcross seed set (percent):			
Mean	28	40	64
Range	0–75	4–81	9–83
Number of plants	66	75	
Self seed set (percent):			
Mean	6	24	39
Range	0–75	0–72	0–70
Number of plants	58	71	62

The range of chromosome numbers in the RBC^2 and RBC^3 generations was much smaller than for the BC^2 and BC^3 generations. Only 3 chromosome numbers were observed, 41, 42, and 43 (table 3, fig. 2, H). The average number of chromosomes for the RBC^2 and RBC^3 generations was slightly greater than 42. Thus, backcrosses in which the BC^1 plants were pollen parents resulted in a much more rapid return to the chromosome number of *vulgare* than did backcrosses with *vulgare* as the pollen parent.

In the BC^1F_2 and BC^2F_2 generations, the chromosome numbers clustered closer to 42 than in the comparable BC^2 and BC^3 generations. The average number of chromosomes in these generations was possibly slightly higher than in their backcrossed counterparts. This implies that a system of self-pollination in BC^1 plants might result in a slightly more rapid return to the chromosome number of *vulgare* than backcrosses, at least in the early generations.

Regression in meiotic behavior toward the recurrent parent was much more rapid in the RBC^2 and RBC^3 generations than in the comparable BC^2 and BC^3 generations. Even after three backcrosses, however, two with hybrid plants as the pollen parents, only about one-half of the population fell within the range of pairing of *vulgare*.

The meiotic behavior of BC^1F_2 and BC^2F_2 plants was as regular if not more regular than that of the comparable BC^2 and BC^3 generations (table 3).

Both backcrossed and selfed populations from backcross plants appeared to follow Kihara's scheme (p. 34). Variability in pairing was such that the most usual number of paired and unpaired chromosomes could not be easily established. However, when the mean number of paired and unpaired chromosomes (based upon analyses of 10 P. M. C.) was determined for each plant, it was observed that the sum of the bivalents and univalents was always equal to or exceeded 21. The average number of bivalents plus univalents in all backcrossed and selfed generations also was equal to or exceeded 21 (table 3). Thus, although monosomic plants were viable, there apparently was strong selectivity against nullisomic plants.

A further measure of cytological stability was obtained from the number of micronuclei per quartet of microspores and the number of quartets which had no micronuclei (table 3). BC^1 plants varied from an average of less than one micronucleus per quartet to an average of nearly six micronuclei per quartet. There was a steady decrease in the frequency of micronuclei with further backcrosses. Again, the return toward the recurrent parent was more rapid in the RBC^2 and RBC^3 generations than in the BC^2 and BC^3 generations. The limited data show that BC^1F_2 and BC^2F_2 plants had approximately the same number of micronuclei on the average as the BC^2 and BC^3 plants.

Large differences were found between plants of the several backcrossed generations in the amount of good pollen (table 3). Most BC^1 plants had a preponderance of aborted or incompletely developed pollen grains, and the anthers failed to open at maturity. A few BC^1 plants, however, fell within the range of variation of *vulgare* in percentage of good pollen. The amount of good pollen increased with further backcrosses until a majority of the BC^3 plants fell within the range observed for *vulgare*. Plants of the RBC^2 generation had an average of 75 percent of good pollen, exceeding the BC^2 and even the BC^3 generation. No data on pollen were obtained for the RBC^3

generation, but all plants had well-filled anthers that apparently dehisced normally.

The fertility of BC [1] plants was in general low (table 3). When 2 or more spikes of 58 different BC [1] plants were selfed, 30 of the plants failed to set any kernels and only rarely were more than 3 selfed kernels obtained from any one plant. In all, 157 kernels were obtained from 2,828 florets (6 percent seed set). When allowed to open-pollinate, the seed set was somewhat higher (9 percent), and only 10 plants of 66 set no kernels. Only 2 plants of 66 failed to set any kernels when backcrossed; hand-pollination of 2,288 florets yielded 648 kernels (28 percent seed set).

When pollen from 15 different BC [1] plants, selected for relatively large amounts of pollen, was used to pollinate florets of *vulgare*, no kernels were obtained with pollen from 3 plants, but the other 12 gave from 1 to 14 kernels per spike of 20 to 28 florets.

In general, BC [2] plants were more fertile than BC [1] plants (table 3). Of the 79 plants which were bagged to test self-fertility, 25 failed to set any kernels. In all, 530 kernels were obtained from 2,221 florets (24 percent seed set). Only 6 of 199 BC [2] plants, or 3 percent, failed to set any kernels when allowed to open-pollinate. The average fertility of these 199 open-pollinated plants was 38 percent (table 3). Seed set from backcrossing was 40 percent, or approximately the same as from open-pollination. Seventy-six BC [2] plants were backcrossed and none failed to produce kernels, although some were relatively low in fertility. The fertility of open-pollinated RBC [2] plants was rather uniformly high, 77 percent on the average. The range in fertility of BC [1] F₂ plants appeared to be the same as for the BC [2] generation (table 3).

Data for individual plants revealed that not until the BC [3] generation were individuals obtained which had 42 chromosomes that appeared to fall within the range of variation of *vulgare* in meiotic behavior, number of micronuclei, percentage of good pollen, and seed set. Because the data from individual plants were limited, the measurement of these factors was not sufficiently precise to determine with certainty that an individual had achieved the stability of *vulgare*. However, 4 BC [3] plants out of 68, 3 BC [4] plants of 19, and 5 RBC [3] plants of 13 appeared to meet these requirements. It is possible that more extensive data would have revealed cytological differences between these plants and *vulgare*.

A simple method of describing the mean cytological regression toward *vulgare* through four backcrosses is provided by the data presented in table 4. It is apparent that each backcross shifted the population approximately one-half the distance toward the recurrent parent when *vulgare* was the pollen parent. This relationship applied rather closely for several characters including chromosome number, closed bivalents, bivalents, univalents, mean number of micronuclei, percentage of quartets with no micronuclei, and percentage of good pollen. It is of interest to note that the genetic trend of a hybrid toward the recurrent parent upon continuous backcrossing in plants in which all chromosomes pair is also one-half per generation. The cytological regression toward *vulgare* was somewhat more rapid when backcross plants were the pollen parents in further backcrosses and is not well described by the above relationship.

TABLE 4.—*Cytological trend toward the recurrent parent compared with trend expected under the hypothesis that the difference between hybrid and recurrent parent is reduced one-half per generation of backcrossing*

Cytological character	Unit	F_1	BC^1	BC^2	BC^3	BC^4	*Vulgare*
Chromosomes	Number expected	35	38.5	40.3	41.1	41.5	42
	Number observed	35	39.1	40.6	41.2	41.6	42
Closed bivalents	Number expected		11.3	15.3	17.3	18.3	
	Number observed	3.2	10.1	13.5	17.1	18.2	19.3
Bivalents	Number expected		14.6	17.7	19.3	20.1	
	Number observed	8.3	14.6	17.4	19.5	20.0	20.9
Univalents	Number expected		7.5	3.8	2.0	1.0	
	Number observed	14.8	7.6	4.4	1.7	.7	.1
Micronuclei per quartet	Number expected		2.6	1.1	.6	.4	
	Number observed	5.0	2.8	1.6	.9	.3	.2
Quartets with no micronuclei	Percent expected		47	70	81	87	
	Percent observed	1	15	33	51	80	92
Good pollen	Percent expected		43	64	74	79	
	Percent observed	2	23	51	65		84

CRITERIA FOR SELECTION

In early backcross generations there was little relationship between the cytological behavior of a particular plant and the cytological behavior of its offspring. For example, one BC^1 plant which had 42 chromosomes most frequently arranged as 12 IIc, 5 IIo, 5 I, and 1 III produced backcrossed offspring which varied from 40 chromosomes (9 IIc, 5 IIo, 9 I, and 1 III) to 43 chromosomes (17 IIc, 3 IIo, and 1 III). Not until an individual had 19 or more bivalent chromosomes was a similar or greater number of bivalents observed consistently in its offspring. This indicates that there is little to be gained toward increasing meiotic regularity by selecting for high pairing until at least 19 pairs have been achieved. It was not until the third or fourth backcross generation (table 3) that most plants reached this degree of meiotic stability.

Low frequency of univalents and of micronuclei similarly were not good criteria for selection in the early generations.

Fertility is relatively easily determined and if selection of the more fertile early-generation plants were effective in raising the general level of fertility, it would be a desirable criterion for selection. This was tested by arranging parental plants according to fertility percentages and tabulating the mean fertility and range in fertility of the offspring of each fertility class (table 5). The limited data indicate

TABLE 5.—*Relationship between the seed set of parental plants and the seed set of their backcrossed offspring*

BC¹ plants		BC² plants		
No.	Seed set in class centers [1]	No.	Seed set	
			Average [2]	Range
	Percent		*Percent*	*Percent*
6	5	8	11.6	1–28
11	15	59	45.4	0–85
6	25	31	40.5	2–86
1	35	8	15.6	2–48
2	45	22	30.3	4–66
1	55	36	51.7	5–91

[1] Seed set when backcrossed to *vulgare*.
[2] Open-pollinated seed set.

that progeny of the more fertile plants varied as widely in fertility as the progeny of less fertile plants; also there was no consistent trend in mean fertility. Observation of numerous families for which no numerical data are available corroborate the data of table 5. This suggests that in the early generations selection of only the more fertile plants is not particularly effective in increasing the fertility of the offspring.

THE BACKCROSS (VT)T

When 312 florets of F_1 plants were pollinated with pollen of *timopheevi*, 11 kernels were obtained (3.5 percent seed set). Seven of the kernels germinated to give plants that either resembled *timopheevi* closely, or were intermediate between *timopheevi* and the F_1 in morphological appearance.

A cytological examination was made of 3 of these plants. Two *timopheevi*-like individuals each had 29 chromosomes. The most usual pairing arrangement in both plants was 8 IIc, 3 IIo, and 7 I. An occasional trivalent was also observed. The third plant was intermediate between the F_1 and *timopheevi* in appearance and had 31 chromosomes, usually associated as 8 IIc, 9 IIo, and 7 I, plus a small fragment. The fragment associated with no other chromosome.

The 7 (VT)T plants were of varying fertility, and a few kernels were obtained from each when allowed to open-pollinate. Nineteen open-pollinated progeny were obtained, all of which resembled *timopheevi* very closely in morphological appearance. A cytological examination was made of 3 of these plants, each of which had 28 chromosomes usually conjugated as 11 IIc and 3 IIo, but occasional univalents were seen. All plants were highly fertile when allowed to open-pollinate. Apparently the return toward *timopheevi*, both morphologically and cytologically, was very rapid following one backcross to that species.

THE F² AND F³ GENERATIONS

The 5 selfed kernels from F_1 plants (p. 37) were sown in the field in 1943. All germinated and grew into mature plants. One of these plants, designated F^2–1, resembled the parental common wheat variety very closely, including its susceptibility to stem rust. It had 42 chromosomes which paired fairly regularly (table 6), and 83 percent of the florets set seed. The following season 18 progeny of this plant were grown. They also closely resembled the parental common wheat variety and all were highly fertile.

The 4 other F_2 plants, designated F_2–2, F_2–3, F_2–4, and F_2–5, differed greatly from one another. All 4 plants had the general gross morphological appearance of *timopheevi*, but they possessed thickened plants parts which often characterize autopolyploids. Plants F_2–2, F_2–4, and F_2–5 had 45, 46, and 49 chromosomes respectively. Conjugation was irregular, univalents and multivalents occurring frequently (table 6). Plant F_2–3 was completely sterile. The other 3 plants set a few kernels when allowed to open-pollinate but they set no kernels under bags. All of the kernels obtained from the 3 partly fertile plants were sown in 1944 and 22 F_3 plants were obtained. These F_3 plants varied amongst themselves even more widely than had their parents, but again all the plants had the gross

general morphological appearance of *timopheevi* (fig. 6, *B* and *C*). Some had thickened plant parts (fig. 6, *B*), whereas others were quite slender and stunted (fig. 6, *C*). All were highly sterile. Seven of these F₃ plants that were examined cytologically were found to have the high chromosome numbers and irregular meiotic behavior that characterized the F₂ (table 6).

TABLE 6.—*Distribution of chromosomes according to type of association in plants of the F₂ and F₃ generations from Triticum vulgare × Triticum timopheevi*

F² GENERATION (from selfed F₁ plants)

Plant	Average and range	2n chromosomes	Type of chromosome association							
			IIc	IIo	II	I	III	IV	V	VI
		Number	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
F₂-1	Average	42	16.6	3.2	19.8	2.0	0	0.1	0	0
	Range		12–21	0–6	18–21	0–6	0	0–1	0	0
F₂-2	Average	45	12.9	3.7	16.6	6 4	1.0	0.6	0	0
	Range		11–16	0–7	13–20	4–11	0–2	0–2	0	0
F₂-4	Average	46	11.1	4.3	15.6	7.9	1.3	0.7	0	0.1
	Range		9–15	2–6	12–18	2–12	0–2	0–2	0	0–1
F₂-5	Average	49	9.6	5.2	14.8	10.1	2.2	0.4	0 1	0 1
	Range		6–14	3–9	9–20	6–15	0–4	0–1	0–1	0–1

F₃ GENERATION (from open-pollinated F₂ plants)

Plant	Average and range	2n chromosomes	IIc	IIo	II	I	III	IV	V	VI
F₂-2-3	Average	45	9.5	2.6	12.0	2.9	2.0	0.5	0	0
	Range		6–14	3–9	10–18	4–9	0–5	0–1	0	0
F₂-2-8	Average	45	15.0	3.5	18 5	3.5	0.5	0.8	0	0
	Range		11–19	2–6	15–21	2–4	0–1	0–2	0	0
F₂-4-4	Average	43	12.3	5.5	17.8	2.5	1.0	0	0	0
	Range		10–16	3–9	16–19	2–4	1–1	0	0	0
F₂-4-5	Average	45	9.5	6.8	16.3	8 2	1.0	0	0 3	0
	Range		6–14	4–9	12–19	6–10	0–2	0	0–1	0
F₂-4-7	Average	46	14.5	4.5	19 0	3.0	0.6	0.4	0.2	0.1
	Range		12–17	3–7	17–21	0–5	0–1	0–1	0–1	0–1
F₂-5-1	Average	41	15.3	4.1	19.4	1.2	0	0	0	0
	Range		12–18	2–8	18–20	1–5	0	0	0	0
F₂-5-2	Average	50	11.3	5.8	17.1	4 2	1.5	1.2	0	0
	Range		7–14	4–9	14–20	2–7	0–2	0–2	0	0

The origin of the F₂ kernels may be inferred from the type of gametes produced by the F₁ plants in reciprocal backcrosses to *vulgare*. It has been shown that the functional pollen of the F₁ had 14 chromosomes and that the functional eggs had 15 to 21 chromosomes. Thus self-fertilized kernels from the F₁ would be expected to receive 14 chromosomes from the pollen and 15 to 21 chromosomes from the eggs, making a total of 29 to 35 chromosomes. Morphologically they would be expected to resemble *timopheevi* or to be intermediate between *timopheevi* and the F₁. The plants obtained deviated widely from the types expected from self-fertilization in F₁ plants, both morphologically and cytologically. Consequently some explanation other than self-fertilization by reduced gametes must be sought to account for these kernels.

The single *vulgare*-like plant had 0 to 6 univalent chromosomes (mean of 2) and occasionally formed the maximum of 21 closed pairs. It could have resulted from the fertilization of an egg which received nearly exactly the 21 chromosomes of *vulgare* by a pollen grain of similar constitution. However, it was not possible to demonstrate by fairly extensive backcrossing that functional 21 chromosome pollen grains were formed by the F₁. Because the anthers of the F₁

FIGURE 6.—Spikes: *A, timopheevi; B,* F$_2$–4–7, a 46-chromosome plant· *C,* F$_2$–2–3, a 45-chromosome plant; *D, vulgare.*

were leathery and tough and did not dehisce, escape of the occasional "good" pollen grains seems unlikely. The restitution (doubling) of a reduced female gamete with 21 *vulgare* chromosomes (or nearly so) and subsequent parthenogenetic development is an alternative hypothesis that is more plausible.

The other 3 F_2 plants had about twice as many bivalent chromosomes and much more frequent multiple associations than did the F_1 plants. The restitution of female gametes after a reduction division involving the loss of several chromosomes, mostly chromosomes of *vulgare*, is a possible explanation which is in accord with the pairing observed (table 6). Another possible explanation involves the union of an unreduced or partly reduced female gamete with a 14-chromosome male gamete of the F_1. The union of a 14-chromosome male gamete with an unreduced egg (35 chromosomes), or with partly reduced eggs that contained 32 or 31 chromosomes, would give plants with 45, 46, and 49 chromosomes, the numbers observed in plants F_2-2, F_2-4, and F_2-5. These plants would have the diploid *timopheevi* complement plus a monoploid *vulgare* complement (minus 3 and 4 *timopheevi* or *vulgare* chromosomes in plants F_2-2 and F_2-4). The pairs would probably be 14 bivalents from *timopheevi* chromosomes depending on the chromosomes that were "minus," plus occasional bivalents from autosyndetic conjugation of *vulgare* chromosomes and multivalents from combined auto- and allosyndesis.

The gross resemblance of the open-pollinated F_3 plants to *timopheevi* suggested that they resulted from the fertilization of partly reduced eggs with high chromosome numbers by pollen grains from nearby plants of *timopheevi*. They could possibly, however, have come from restitutions and parthenogenetic development of partly reduced eggs, or from self-fertilization.

THE F_2 (O. P.) GENERATION OR FIRST NATURAL BACKCROSS GENERATION

In the summer of 1942, a row of F_1 plants was grown between rows of the 2 parents. From 329 open-pollinated spikes of the F_1 plants 75 kernels were obtained. Because the F_1 was late in maturity, its flowering period coincided more closely with that of *timopheevi* than with that of the earlier maturing *vulgare* parent. It was not unexpected, then, that 25 of the 30 plants obtained from these kernels, which were almost certainly from outcrosses, resembled *timopheevi*. The variation in appearance of these 25 natural backcrossed plants was the same as that of plants from controlled backcrosses to *timopheevi*. Of the remaining 14 F_2 (o. p.) plants, 8 were intermediate between the F_1 and *timopheevi* but more closely resembled the F_1, and 6 resembled *vulgare*. The former probably were from eggs which had largely a *vulgare* complement of chromosomes fertilized by male gametes from *timopheevi*. The latter were probably from natural backcrosses to *vulgare*.

During the 1943 season the following variations were made in order to obtain a larger proportion of *vulgare*-like F_2 (o. p.) plants. About 100 F_1 plants were interplanted with the variety Marquis. By delaying the planting date of Marquis and cutting it back, heading was made to coincide with that of the F_1 plants; 174 F_2 (o. p.) kernels were found on the F_1 plants. A random group of 84 was sown in 1944 and 31 plants were obtained. Twenty-five individuals with awnlets like those of Marquis were observed, providing evidence that natural outcrosses to Marquis gave rise to the majority of the kernels (fig. 7, B). Four plants which resembled the common wheat parent were found (1 shown in fig. 7, D), 1 *timopheevi*-like plant (fig. 7, C), and 1 plant

FIGURE 7.—Spikes: *A*, 28-chromosome *timopheevi*-like F_3 (o. p.) plant; *B*, awn-leted F_2 (o. p.) plant with 38 chromosomes, probably from an outcross to Marquis; *C*, F_2 (o. p.) plant with 30 chromosomes, probably from an outcross to *timopheevi*; *D*, F_2 (o. p.) plant with 40 chromosomes, probably from an out-cross to the common wheat parent; *E*, F_1-like F_3 (o. p.) plant with $2n=21$ chromosomes, probably parthenogenetic in origin.

which was intermediate between the F_1 and *timopheevi*. Since the common wheat parent and *timopheevi* were growing within a few feet of some of the F_1 plants, occasional backcrossing to them probably explains the few plants which did not resemble Marquis. These results were very different from the results of 1942 when the F_2 (o. p.) population was largely *timopheevi*-like, and serve to emphasize the control of natural backcrossing that is possible.

A cytological examination was made of 20 of the 31 F_2 (o. p.) plants which were grown in 1944. The single *timopheevi*-like plant (fig. 7, *C*) and the plant intermediate between the F_1 and *timopheevi* each had 30 chromosomes. The usual pairing for both plants was 8 IIc, 5 IIo, and 4 I. Chromosomes numbers of the 18 *vulgare*-like plants which were examined cytologically are given in table 3. The distribution of chromosome numbers appeared to be about the same as for the BC^1 generation. Similarly the variation in numbers of closed bivalents, total bivalents, and univalents approximated the values for the BC^1 generation (table 3).

The fertility of the F_2 (o. p.) plants varied considerably and was correlated with morphological appearance. Plants which resembled the F_1 were completely sterile. Nearly half of the *vulgare*-like and *timopheevi*-like plants, however, set some kernels. The range in fertility of *vulgare*-like F_2 (o. p.) plants was approximately the same as that of the comparable BC^1 generation..

THE F_3 (O. P.) GENERATION

The appearance of F_3 (o. p.) plants was related to the appearance of their F_2 (o. p.) parents. *Timopheevi*-like F_2 (o. p.) plants had F_3 (o. p.) offspring which usually resembled *timopheevi* but occasionally were like the F_1 in appearance. Eight such F_3 (o. p.) plants were examined cytologically. Five *timopheevi*-like plants (1 shown in fig. 7, *A*) had 28 chromosomes each and 1 plant had 29. The usual pairing was 14 bivalents in the 28-chromosome plants and 14 bivalents plus 1 univalent in the 29-chromosome plant, although some univalents were observed in all 5 plants. One F_1-like plant had 35 chromosomes which usually associated as 7 IIc, 6 IIo, 6 I, and 1 III. This plant probably resulted from an outcross with *vulgare*. Another F_1-like plant (fig. 7, *E*) had only 21 chromosomes, usually associated as 2 IIc, 3 IIo, 8 I, and 1 III, and probably was of parthenogenetic origin.

Progeny of *vulgare*-like F_2 (o. p.) plants usually resembled *vulgare* more closely than did their parents, but occasional plants resembled the F_1. *Vulgare*-like F_3 (o. p.) plants probably resulted from selfing of F_2 (o. p) plants or from outcrosses to the common wheat parent or Marquis. F_1-like plants probably were from outcrosses to *timopheevi*. The chromosome numbers and meiotic behavior of 11 *vulgare*-like F_3 (o. p) plants are given in table 3. The cytological regularity was about the same as that of the corresponding BC^2 generation.

In the F_2 (o. p.) and F_3 (o. p.) generations, all *timopheevi*-like or F_1-like plants were highly resistant to stem and leaf rust. Segregation for stem rust resistance occurred, however, among the *vulgare*-like individuals of these generations. Of 6 *vulgare*-like F_2 (o. p.) plants grown in 1943, 3 were highly resistant and 3 were intermediate; in

1944 there were 5 resistant, 12 intermediate, and 12 susceptible plants. Three *vulgare*-like F₃ (o. p.) families grown in 1944, all from highly resistant F₂ (o. p.) plants, segregated as follows (resistant: intermediate: susceptible): 22:3:4, 50:0:11, and 4:0:3. Thus there was no more difficulty in maintaining stem rust resistance in a large proportion of the natural backcrossed than in the artificially back-crossed populations.

A few of the stem and leaf rust resistant F₃ (o. p.) plants were selected and open-pollinated progeny grown for two additional gener-ations. Although there was no difficulty in fixing rust resistance in highly fertile lines, few agronomically desirable types were obtained. Hybrids were then made between the better lines and several common wheat varieties and the F₁'s were backcrossed to the common wheat varieties one or more times. Segregates from these back-crosses resembled the recurrent parent agronomically. Far less effort in transferring stem and leaf rust resistance to agronomically desir-able types was required by this system than by backcrossing in early generations when fertility was poor.

THE BC²F₄ GENERATION

Cytological observations were made on one of the most promising BC²F₄ lines. It bred true for resistance to both leaf and stem rusts and to mildew, was completely fertile, and of fair agronomic type. Six plants were examined, all of which had 21 pairs of chromosomes (fig. 2, *I*), and 96 percent or more of the quartets were free from micronuclei. This line appeared to be as stable cytologically as the *vulgare* parent. In its hybrids with Marquis and Reward, 21 pairs were observed uniformly, and 97 percent or more of the quartets were free from micronuclei in 3 F₁ plants of each hybrid. Thus the rust and mildew resistance of *timopheevi* can be incorporated into cytologically stable 21-chromosome wheats. Moreover, because these derivatives from *timopheevi* are cytologically regular in hybrids with certain *vulgare* wheats, there should be no cytological difficulties if they are used as disease-resistant parents.

DISCUSSION

The special position that *Triticum timopheevi* occupies among the 28-chromosome wheats, first recognized from cytological evidence, is further substantiated by the breeding behavior of this species in its hybrid with *T. vulgare*, agricultural variety 2666A2-2-15-6-3. This hybrid does not follow Kihara's (*18*) scheme established from re-searches upon other pentaploid wheat hybrids in which the parental chromosome numbers are regained following selfing of the hybrid. Rather the hybrid of *T. vulgare* × *T. timopheevi* is probably completely male-sterile in nature as a result of indehiscence of the anther, and the few kernels obtained apparently are produced by other than the usual sexual process. However, once the population had been shifted toward either parent by backcrossing with the F₁ as the female, it was found that the return to the 42-chromosome or 28 chromosome condition was according to Kihara's scheme.

This hybrid also differs significantly from other pentaploid wheat hybrids in the character of the populations obtained from back-crossing to the common wheat parent. In other pentaploid hybrids

all chromosome numbers from 14 to 21 are represented in functional male gametes of F_1 plants, although the most numerous functioning gametes have chromosome numbers approaching one or the other parent (*34, 36*). It was demonstrated in the present study that the only male gametes that functioned possessed 14 chromosomes. As a consequence no progress toward the *vulgare* type was noted from backcrosses made with the F_1 as the pollen parent. When the *timopheevi-vulgare* F_1 was backcrossed with pollen from *vulgare*, the results diverged less from other pentaploid hybrids than in the reciprocal backcross, but fewer pairs of chromosomes were observed and the average fertility of the backcrossed population was lower. The relatively remote relationship between *timopheevi* and common wheat is thus expressed in their hybrid in low chromosome pairing and low fertility, expecially in the male gametes. Both factors serve as major barriers to the transfer of genes.

Nevertheless, transference of genes governing stem and leaf rust resistance by successive backcrosses was obtained. However, only a few genes govern rust resistance and these genes were transmitted with approximately equal frequency through both male and female gametes of early-generation backcross plants. Because functional male gametes of backcross plants were genetically and cytologically similar to *vulgare* gametes, backcrosses in that direction acted as a fine sieve which passed genes controlling rust resistance but screened out nearly all other *timopheevi* genes. Accordingly, it was possible to fix stem and leaf rust resistance rapidly in cytologically stable 42-chromosome types by backcrosses with early generation backcross plants as pollen parents.

It is possible that genes governing stem- and leaf-rust resistance are located in one or more of the few *timopheevi* chromosomes which appear to have nearly exact homologues in *vulgare*. Presumably such chromosomes could be substituted for their homologues without seriously decreasing the viability of either male or female gametes of backcrossed plants. Because mildew resistance was found in lines derived from this type of backcross, the genes concerned can also be presumed to be located in chromosomes of *timopheevi* which have homologues in *vulgare*.

Other characters of *timopheevi* studied, including bunt resistance, were less easily transferred to *vulgare* types. If the genes concerned were located in *timopheevi* chromosomes which pair sporadically with chromosomes of *vulgare*, the gametic unbalance and consequent gametic elimination which inclusion of partial homologues might cause may account for the failure of such characters to appear in backcrossed generations. Another possible explanation is that these characters were governed by numerous genes. Anderson (*2*) has discussed the reasons why transfers of characters from one species to another are likely to be difficult when a character is controlled by several genes.

The transfer of genes in chromosomes which completely lack homology depends upon the substitution or addition of a complete chromosome pair, as demonstrated by Gerstel (*6, 7*). The place of "alien addition" and "alien substitution" races in practical plant breeding remains to be determined.

Although stem-rust resistance was transferred to apparently stable 42-chromosome *vulgare* types by successive backcrosses, utilization of

natural backcrosses proved more efficient in time and labor for this transfer. Because the F_1 hybrid was nearly completely male-sterile under field conditions, any kernels produced upon F_1 plants most likely were from outcrosses. By interplanting with the desired recurrent parent and with proper isolation, naturally backcrossed kernels were obtained more easily than by hand-pollinations. A mixture of self-pollinated and backcrossed kernels was obtained when these seeds were again interplanted with the recurrent parent. Among these plants were numerous rust-resistant individuals of moderate to high self-fertility. However, undesirable characters, such as lateness of maturity and weak straw, were usual and few or no agronomically desirable lines could be isolated by selection in 2 additional open-pollinated generations. Further improvement was accomplished by artificial backcrosses to common wheat varieties. Under the system outlined above, natural backcrossing was utilized in early generations to lessen the labor required; while artificial backcrosses were employed when fertility had been restored and hand-pollinations were more fruitful.

SUMMARY

In the hybrid between *Triticum vulgare* and *T. timopheevi* there were marked differences in chromosome numbers and pairing, fertility, and morphological appearance in generations derived by selfing and reciprocal backcrosses to *vulgare*. This hybrid differs significantly from other pentaploid hybrids in its breeding behavior.

The rare kernals produced upon selfed F_1 plants appeared to have arisen by an abnormal sexual process.

Backcrosses obtained by using pollen of the F_1 on *vulgare* were nearly indistinguishable from the F_1 in morphological appearance cytological behavior, and fertility.

Plants obtained by using pollen of *vulgare* on the F_1 were variable but in general intermediate between the F_1 and *vulgare*. The resemblance to *vulgare* increased through four similar backcrosses. The mean difference in cytological behavior and fertility between plants of each backcrossed generation and *vulgare* was decreased by half per generation of backcrossing to *vulgare* when backcrossed plants were the seed parents.

When the second backcross was made with the first backcross plants as the pollen parents, the resulting population resembled *vulgare* more closely than did the reciprocal backcross and was more regular cytologically and more fertile. Resistance to stem and leaf rusts and mildew was maintained in this type of backcross, but nearly all other characters of *timopheevi*, including resistance to bunt, were eliminated. This system of mating thus allowed a rapid transfer of *timopheevi* resistance to stem and leaf rusts and mildew to 42-chromosome *vulgare* types.

Little was gained toward greater fertility or meiotic stability by selecting the more fertile or more cytologically regular plants in early generations. Selection in early generations should be only for the specific genes sought.

The self-sterile F_1 may be satisfactorily backcrossed by planting in proximity to the desired male parent.

Despite the poor pairing and low fertility of the F_1, genes for resistance to stem rust, leaf rust, and mildew possessed by *timopheevi* were

transferred to *vulgare*. This was accomplished most efficiently by three or four generations of natural backcrossing resulting in fertile disease-resistant types which were then backcrossed successively to *vulgare* until the genes for resistance were stabilized in an otherwise *vulgare* genotype.

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RED STEM CANKER OF COWPEA, CAUSED BY PHYTOPHTHORA CACTORUM [1]

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INTRODUCTION

In 1944 a disease not heretofore observed was found causing injury and death to a considerable number of plants in a field of cowpeas (*Vigna sinensis* (Torner) Hassk.) at Experiment, Ga. The following year the disease appeared in another field but it was less destructive, occurring most commonly in low, poorly drained areas. The first year there was no apparent correlation between the prevalence of the disease and the soil moisture; then the disease was present on a hillside where soil and air drainage were excellent. The disease was not observed in 1946 or 1947, probably because few growing cowpea plants were seen and little time was spent searching for the disease. So far as known the disease is not generally destructive, but it is potentially capable of doing considerable damage. This paper reports the cause of the disease and other pertinent facts about it.

SYMPTOMATOLOGY

The disease under discussion is easily recognized by the long, reddish lesions that may occur on any part of the stem or the petioles of cowpeas. This characteristic red color suggested the common name, red stem canker. The canker may involve part or all of the circumference of the stem and may extend from the base to the tip of the plant affected (fig. 1). At first the infected tissue looks water-soaked, but it soon becomes "maroon"[3] or "diamine brown" (*6*).[4] The centers of the old lesions frequently are lighter than the margins; often they are nearly "brick red" or "prussian red," but the center of a single lesion may vary considerably in color. When the tissue dries out the lesions may split longitudinally. Frequently the center of a lesion is invaded by one or more different fungi, largely if not entirely saprophytic.

FIGURE 1.—Cowpea plants naturally affected with the red stem canker: *A–C*, Plants that have become one-sided because of killing of one side by the red stem canker fungus; *D*, plant practically killed. ✕ approximately ⅓.

Infected stems of young plants may be girdled and the plants killed (fig. 2, *B*). Often only one side of a plant is seriously injured and its branches are killed, and thus a one-sided plant is produced (fig. 1, *A–C*). Sometimes the apical bud is killed and elongation of the main stem stops. Usually the lesions appear first a short distance above the soil surface and the base of the stem is not injured. When infection does take place below the surface of the soil, the fungus grows up the stem and produces a narrow lesion which becomes broader when it is above the soil surface (fig. 3).
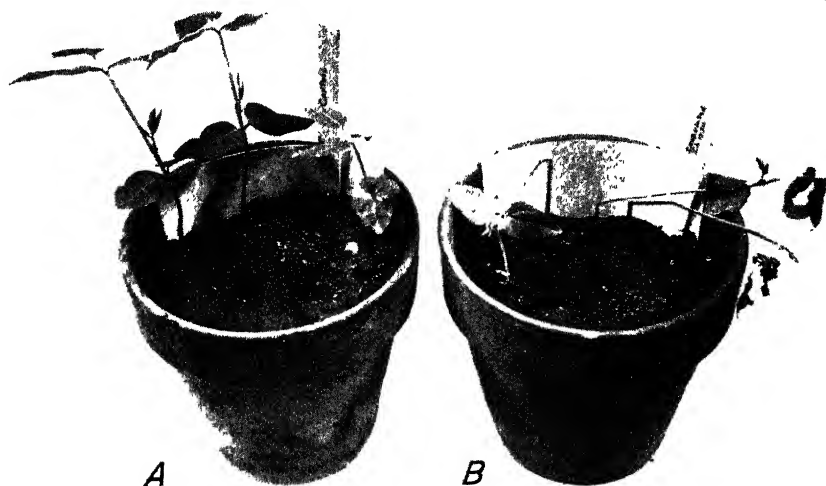
FIGURE 2.—*A*, Uninoculated cowpea seedlings; *B*, seedlings of same age inoculated with the red stem canker fungus and photographed 5 days after inoculation. × approximately ¼.

The symptoms of red stem canker differ from those of bacterial canker as described by Burkholder (*2*) and Dunlap [5] and described and illustrated by Hoffmaster (*4*) in that the lesions of the former are red and are not swollen or cracked. Red stem cankers differ from the lesions produced by *Rhizoctonia* in that the latter are largely underground. Typical cankers caused by *Macrophomina phaseoli* (Maub.) Ashby on cowpea differ from red stem cankers in that they have chocolate-brown borders and ashen centers and their surfaces often are thickly dotted with pycnidia.

ISOLATIONS AND INOCULATIONS

The surface of some lesions shows no change from that of the healthy stem except a uniformly reddish color; therefore, it gives no clue to the probable causal agent. On other lesions there occur lighter areas that are often covered with fungus fruiting bodies.

[5] DUNLAP, A. A. TWO BACTERIAL DISEASES IN TEXAS. U. S. Bur. Plant Indus., Soils, and Agr. Engin., Plant Dis. Rptr. 27: 274. 1943. [Processed.]

FIGURE 3.—Cowpea stems inoculated just below the surface of the soil with the red stem canker fungus. Note that the fungus produced lesions at the points of inoculation and then grew upward forming narrow, reddish lesions that became broader after reaching the above-ground parts of the stems. $\times$ 1⅒.

These may be the imperfect stage of an anthracnose fungus, sometimes a type with curved spores and at other times a type with straight spores resembling those of *Glomerella cingulata* (Ston.) Spauld. and Schrenk. The ascospore stage of the latter fungus is sometimes present. Under field conditions a coating of spores of *Fusarium* sp. also may be found on the older lesions. Less frequently fruiting bodies of other fungi such as *Alternaria, Phoma, Helminthosporium,* or *Macrophomina* are present. Of these fungi the curved-spored *Colletotrichum* and a species of *Fusarium* were found most consistently. These two fungi were isolated, but inoculation experiments showed that they were nonpathogenic. Further search showed that oospores of a phycomycetous fungus were consistently present in the diseased tissue; this fungus was then isolated and used in inoculation experiments.

On December 21, 1945, five cowpea plants of an unidentified variety growing in pots in the greenhouse were inoculated with this phycomycetous fungus by placing bits of agar on which it was growing against the unwounded stem near the surface of the soil; then the pots were held in a moist chamber for 70 hours. Control plants were treated in the same manner except that sterile agar was placed against the stems. The five inoculated plants were all dying 4 days after inoculation, the stems being nearly girdled at the point of inoculation. The fungus had penetrated the tissue in both directions from the point of infection. There was no injury to the control plants. The fungus used in the inoculations was reisolated from the diseased tissue.

The phycomycetous fungus used in the experiment just described and the reisolate were used in a second inoculation experiment begun on January 4, 1946. Three plants were inoculated with each fungus, and three others were held as controls. The fungus growing on agar was placed against the stem of each seedling a short distance above the soil surface. Sterile agar was placed against the stems of the control plants. All plants were held in a moist chamber for 68 hours. Four days after the inoculations were made five of the six inoculated plants had stem cankers typical of those seen under field conditions. Five days after inoculation the stems of the three plants inoculated with the original isolate were so badly decayed that their tops had fallen over (fig. 2, *B*). The fungus under investigation was reisolated from plants in both sets of inoculations. The tissue of most of the lesions contained oospores typical of the fungus used in making the inoculations. Thus the disease was reproduced by the fungus isolated, the fungus was again recovered, and this reisolate reproduced the disease and was recovered.

In a third inoculation experiment begun on March 11, 1946, 12 plants were inoculated with the original isolate and 10 of them became infected. At the same time 12 plants were inoculated with the reisolate from the second experiment and all of them became infected.

These experiments and a number of others in which plants were inoculated to test varietal susceptibility proved conclusively that the phycomycetous fungus is the cause of the disease. Infection usually was evident 3 or 4 days after inoculation. Seedling cowpea plants were most susceptible, but the youngest tissues of older plants, even those setting seed, were susceptible also. Some of the lesions a

week old were 2 to 3 inches or more in length and often involved the entire diameter of the stem so that it broke over.   In other cases the lesions remained superficial.   Figure 4 shows lesions on cowpea stems inoculated with a reisolate of the original culture.   At first the lesions were reddish green.   The amount of red varied with the lesion, some being "liver brown" and others "yellowish olive."   Often affected plants that were not girdled and killed were more or less dwarfed or unsymmetrical.   Some stems had lesions 5 to 8 inches long 18 days after inoculation.

In order to determine the relative resistance of different varieties of cowpea to the phycomycetous fungus being studied, several varieties were grown in pots in sterilized field soil in the greenhouse and inoculated as described previously when the seedlings were putting out their first trifoliate leaves.   As shown in table 1, a high percentage of infection of most of the varieties tested was obtained.   Alalong, Speckled Crowder, and Purple Hull appeared to be somewhat resistant in these small-scale tests.   None of the 31 control plants of 8 varieties became infected.

TABLE 1.—*Results of inoculating various varieties of cowpea with the red stem canker fungus*

Variety	Plants inoculated	Plants infected	Variety	Plants inoculated	Plants infected
	Number	Percent		Number	Percent
Alabrowneye	7	71	Cream Crowder-Lady	8	100
Alabunch	15	93	Cream-longpod	10	100
Alalong	8	25	Early Dixie Queen	8	100
Blackeye Wilt-resistant	7	100	Jackson 21	4	100
Blue Goose	13	92	Purple Hull	3	33
Brabham	17	100	Purple Hull Bunch	14	100
California Blackeye No. 5	3	67	Sixweeks	15	93
Chinese Red	16	75	Speckled Crowder	15	27
Cream	1	100	Sugar Crowder	19	90
Cream Crowder	3	67	Whippoorwill	8	100

CULTURAL CHARACTERISTICS AND MORPHOLOGY OF THE FUNGUS

The causal fungus is easily isolated from the young red stem cankers by ordinary cultural methods, but this is no longer true after the tissue has been invaded by bacteria and secondary fungi.   The fungus grows well on most media.   The mycelium is white or slightly brownish on some media and varies in luxuriance with the medium.   Oospores are formed much more abundantly on corn-meal agar than on oatmeal agar.   Moderately good growth takes place on Leonian's malt agar, but oospores are formed sparsely on it.

Usually on the above-mentioned agars the hyphae range from 4μ to 4.5μ in diameter, but sometimes hyphae of irregular diameter 8μ or more across occur.   There also are numerous short hyphal branches 5μ to 15μ long.   Young branches may be greatly constricted where they join the mother hypha, then become broader than the latter a short distance from it, and taper gradually to a blunt point.

When a small piece of agar on which the fungus is growing is placed in water sporangia are formed on sporangiophores which are simple at

FIGURE 4.—*A*, Uninoculated stem of cowpea; *B* and *C*, cowpea stems inoculated with the red stem canker fungus in the greenhouse and photographed 8 days after inoculation. × 1¼.

first but later branched and vary in length from 50μ to $1,000\mu$. When a sporangium is to all appearances mature a secondary sporangiophore emerges from the first a short distance below the sporangium. This secondary sporangiophore elongates until it reaches a short distance above the primary sporangium, and then it forms a secondary sporangium. This process continues until four or more sporangia are produced, each a short distance above the other. The sporangia thus produced are lemon-shaped, oval, or elliptical and have a distinct papilla, usually terminal but sometimes lateral. Fifty sporangia measured 25μ to 50μ in diameter, but more commonly they ranged from 30μ to 40μ.

Germination in water is by formation of zoospores. These are completely formed within the sporangium and emerge from it in a mass but scatter almost immediately. Frequently several zoospores fail to escape through the apical pore with the others. Some of these escape later, but others never do. Usually the zoospores remain active for about half an hour and then round off; after a short rest period they germinate.

When grown on malt agar, the oogonia are pyriform or subspherical and measure 18μ to 30μ by 21μ to 31μ (50 measured; average, 25.3μ by 26.7μ). The antheridia are largely paragynous, but sometimes amphigynous, and are attached to the lower half of the oogonia. They remain distinct and appear to be empty when the oospores are formed.

The oospores are slightly yellowish to almost colorless. They are subspherical and have a smooth wall 1.0μ to 2.2μ thick. The diameter measurements were as follows:

Source of oospores:	Oospores measured (number)	Range (μ)	Average (μ)
Vigna sinensis	50	13.7 to 23.1 by 13.7 to 25.2	19.6 by 20.2
Oatmeal agar	55	14.7 to 27.3 by 15.8 to 29.4	21.9 by 22.3
Leonian's malt agar	65	14.7 to 27.3 by 14.7 to 27.3	20.3 by 21
Corn-meal agar	50	14.7 to 26.2 by 16.8 to 27.3	20.9 by 21.5

They averaged 20.8μ.

Few chlamydospores were seen.

The effect of temperature on growth of the fungus was determined by growing a single isolate in five petri dishes at each of various temperatures. On Leonian's malt agar the fungus failed to make any growth at 7° to 8° C. in 9 days; its optimum for vegetative growth was between 26° and 28°, although its growth at 31.5° was only a little slower. Oospores were formed more abundantly below 25° than above. The fungus grew well at 31.5°, but it made very slight growth at 35° and died after 3 days. The fungus was also grown on corn-meal agar at certain temperatures. At 30°, 25.5°, and 24.6° the mycelial colonies averaged 44.3, 40.4, and 40.2 mm. in diameter, respectively, in 4 days. The fact that this fungus made fair growth on corn-meal agar in 4 days at temperatures up to 30° is important, because Tucker (8) used the temperature responses of certain species of *Phytophthora* when grown on corn-meal agar at 26° to 28° for 4 days in his key for separating them.

LIFE HISTORY OF THE FUNGUS

Just how the red stem canker fungus attacks plants under natural conditions is not entirely clear. The natural assumption would be that infection takes place in the root or stem beneath the soil surface or near it and that the fungus then gradually progresses up the stem. In many instances, however, there is no evidence to support such an assumption. It appears that frequently infection must take place in the young growing tissues above ground, since there is no evident connection between the lesion and the underground parts of the plant.

An experiment was performed to learn whether infection could take place underground and, if so, whether the fungus could grow up the stem into the above-ground parts. Some soil was removed from one side of the base of young cowpea plants in 4 pots, a small piece of agar on which the red stem canker fungus was growing was placed against the underground part of the stem, and the soil was replaced. Two pots in which sterile agar was placed against the underground stems served as controls. In all, 22 plants were inoculated and 18 were held as controls. Only 4 of the inoculated plants and none of the controls became infected. In a later experiment performed in the same manner 60 percent of the plants inoculated were infected. Evidently, therefore, infection can take place below ground, although under the experimental conditions it took place much more readily above ground. When infection did take place underground, the fungus caused only a very narrow lesion that extended from the point of inoculation to a point well above the surface of the soil and then broadened out and involved a much greater portion of the stem (fig. 3).

The red stem canker fungus can penetrate the cortical cell walls as well as pass between them, especially in young tissue. The hyphae are greatly constricted where they pass through the cell wall. The walls of the affected cells as well as their densely granular contents are reddish. The presence of so much red in the cells imparts this color to the lesions.

When pieces of infected stem are placed in water, hyphae grow out into the water and sporangia are formed. In one instance many sporangia had formed in this manner in 16 hours and some had already germinated. This suggests that a layer of water on a lesion for 16 to 24 hours, and possibly less under conditions very favorable for the fungus, would suffice to enable the fungus to sporulate on the surface of a plant and reinfect it or an adjacent plant to which the zoospores might have been blown or on which they might have fallen.

TAXONOMY OF THE FUNGUS

A careful study of the germination of the sporangia of this cowpea fungus shows that it belongs in the genus *Phytophthora*. This fungus makes good growth on malt, corn-meal, and oatmeal agars after 6 days at 20° C., it produces oospores abundantly on these culture media, the antheridia are predominantly paragynous, and it makes a fair growth on corn-meal agar in 4 days at 26° to 28°. These facts place it in the species *P. cactorum* (Leb. and Cohn) Schroet. according to the key given by Tucker (*8*). The red stem canker fungus

grows at 27°, forms sexual bodies after being transferred from pea broth to distilled water as described by Leonian (*5, p. 29*), and has predominantly paragynous antheridia and papillate sporangia; hence it falls into the species *P. cactorum* in Leonian's key (*5*). The cowpea fungus differs from *P. cactorum* as given in Rosenbaum's key (*7*) only in that its oospores are smaller. The work of Tucker and Leonian, however, make it clear that rather wide variations in the size of the oospores are to be expected. Tucker (*8, p. 150*) gave a résumé of the measurements reported by various writers for oogonia and oospores of *P. cactorum*. Among the measurements given are those by Hartig (*3*), who stated that oogonia in *Fagus* sp. measured about 20μ. Measurements of oospores of *P. cactorum* given by other workers range from 15μ to 45μ.

The cowpea strain of *Phytophthora cactorum* differs from those studied by Leonian (*5*) in temperature response. Leonian stated that 31° C. seems to be the upper limit for the growth of this fungus and at that temperature growth is very poor. He reported that no strain grew at 32.5°. The cowpea fungus made a slight growth at 35°, but it died in 3 days at that temperature. At 31.5° it grew well. Tucker (*8*) considered 35° to be near the extreme limit for survival of *P. cactorum*. This temperature is also near the extreme or possibly a little above that for survival of the cowpea fungus. Tucker's strains of *P. cactorum* made some growth at 5° in 4 days. The strain from cowpea made no growth at 7° to 8° in 9 days. Beach's (*1*) strain of this fungus had a minimum temperature of 7° to 9°, which is about the same as that of the cowpea strain. Since the cowpea fungus varies in only a few minor respects from *P. cactorum* as conceived by most workers and the variations are within the range of variation described by other workers, there seems to be no reason why it should not be placed in this species.

No record of *Phytophthora cactorum* attacking cowpea was found in the literature. This fungus has been reported, however, as being parasitic on several other legumes as well as on a wide range of non-leguminous plants.

SUMMARY

A disease characterized by long, reddish lesions on the stems of cowpea plants and often resulting in the death of a part or all of the affected plant was found at Experiment, Ga. It is described and named red stem canker. A species of *Phytophthora* was isolated from the diseased stems and its pathogenicity was proved. Morphological and physiological studies showed that this cowpea *Phytophthora* falls within the range of the species *P. cactorum* as now conceived by most writers; hence it is considered to be a strain of that species.

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THE GENETICS OF MOSAIC RESISTANCE IN NICOTIANA GLUTINOSA [1]

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Nicotiana glutinosa L. has contributed to the development of varieties of tobacco (*N. tabacum* L.) that, from a practical standpoint, are immune to the tobacco mosaic virus. *N. glutinosa*, when inoculated with the tobacco mosaic virus, reacts with a necrotic spot at each point of entrance of the virus into the leaf, where the virus is usually localized.

Holmes [2] demonstrated that *N. glutinosa* carries a dominant factor pair *NN* for necrotic spotting, and he showed that the factor could be transferred to *N. tabacum*. The writer has developed many varieties of tobacco, including burley, dark air-cured, and dark-fired, that contain the *N* factor and remain completely free from mosaic in the field in spite of inoculation or growing next to affected susceptible plants. Some of these *NN* varieties (Ky 52, Ky 56, Ky 151, and Ky 160) are being grown commercially with complete success as to type of plant, quality of product, yield, and freedom from mosaic. The only injury from mosaic, under field conditions, has been when transplants were inoculated on the stalk with virus from living plants at pulling time, or when the midvein of a leaf was inoculated after rapid growth had commenced. Stalk inoculation resulted in a systemic necrotic disease that quickly killed the plant, while inoculation of the midvein sometimes resulted in a systemic necrotic disease that progressed more slowly but eventually caused death. Under ordinary farm conditions where susceptible varieties become heavily infected, the *NN* varieties show no injury from mosaic even though handled by men who chew or smoke natural leaf tobacco carrying the virus.

In attempting to understand the genetics of mosaic resistance in these varieties, questions arose as to (1) whether *N. glutinosa* would develop mottling in case of failure of the *N* factor to function, and (2) if mottling developed whether susceptibility is governed by a gene separate and distinct from the *N* gene. Or, to state the question another way, is the constitution of *N. glutinosa NN* or *NN AA* so far as its reaction to mosaic is concerned? To answer these questions a breeding experiment was set up in which *N. glutinosa* was crossed with a mosaic-resistant burley in which mosaic resistance had been derived from Ambalema, a mosaic-resistant variety from Colombia, South America. In Ambalema, and in the mosaic-resistant burley

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derived from it, mosaic resistance is governed by two recessive factor pairs a_1a_1 a_2a_2 that are apparently alleles of the factors for susceptibility A_1A_1 A_2A_2 contained in all mosaic-susceptible varieties of *N. tabacum*. If a mosaic-resistant plant carrying the recessive factors a_1a_1 a_2a_2 is crossed with *N. glutinosa* and the hybrid made fertile by doubling the chromosome number, any seedlings from this cross, or from a backcross with mosaic-resistant burley, that prove susceptible to tobacco mosaic must have received the susceptibility factor from *N. glutinosa*.

After the initiation of this work in June 1944 a partial answer to the first question was given in a paper by McKinney and Clayton [3] in which they showed that if, following inoculation with tobacco mosaic virus, a plant of *N. glutinosa* is kept at a temperature of 97° F., typical mosaic mottling will develop. It is evident, therefore, that *N. glutinosa* carries a factor for susceptibility to tobacco mosaic, but this did not answer the question as to whether the reaction to the *N* factor had been changed or whether the *N* factor had simply been temporarily put out of operation, thereby allowing a susceptibility factor of the *A* type to operate.

The hybrid *N. glutinosa NN* × mosaic-resistant burley a_1a_1 a_2a_2 was prepared. It proved to be sterile, as is usual with this interspecific hybrid. Lateral buds were treated with colchicine until finally a branch developed that was fertile. *N. digluta*,[4] as the new species is known, was used as the male parent in a backcross with the a_1a_1 a_2a_2 burley. Seed set in abundance but failed to germinate except for two seeds in a second sowing.[5]

One of the two seedlings of the cross *N. tabacum* a_1a_1 a_2a_2 × *N. digluta NN* a_1a_1 a_2a_2 was grown to maturity. It gave the necrotic reaction and so carried the *N* factor, and was partially fertile. Two hundred five F_2 seedlings were grown of which 24 gave the necrotic reaction and therefore carried the *N* factor, 60 were mosaic-susceptible and therefore were minus the *N* factor and carried at least one *A* or *A*-like factor for mottling, while 205 appeared to be nonnecrotic spotting but were mosaic-resistant and therefore did not carry either the *N* or *A*-like factors but only *a* factors.

The reciprocal cross *N. digluta* × *N. tabacum* produced viable seed. If *tabacum* chromosomes paired only with *tabacum*, and *glutinosa* only with *glutinosa*, all gametes of *N. digluta* should be 24+12 and the progeny of the cross *N. digluta NN* a_1a_1 a_2a_2 × *N. tabacum* a_1a_1 a_2a_2 should be (24+12)+24 and contain the *N* factor from *N. glutinosa*. All seedlings should develop necrotic spots when inoculated, and it should be necessary to grow the second generation of this cross to get the desired results. Actually, the seedlings were not uniform. Of 18

[3] McKinney, H. H., and Clayton, E. E. GENOTYPE AND TEMPERATURE IN RELATION TO SYMPTOMS CAUSED IN NICOTIANA BY THE MOSAIC VIRUS. Jour. Hered. 36: 323–331, illus. 1945.

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[5] An examination showed that the endosperm had failed to develop in the majority of seeds, leaving a hollow shell. The reciprocal in which *N. digluta* was the female parent produced fertile seed. The chromosome number of the endosperm in the sterile cross was, theoretically, 24+24+36=84, while in the reciprocal cross that proved fertile it was 36+36+24=96 chromosomes.

plants grown to maturity and tested for their reaction to the tobacco mosaic virus, 17 were without the dominant *N* factor from *N. glutinosa*. Of the 17, 16 proved to be susceptible to tobacco mosaic virus, as they developed typical mottling, and therefore carried at least one *A* or *A*-like factor. One proved to be resistant to mosaic but did not develop necrotic spotting, indicating that it did not contain either the *N* or *A*-like factors but only the recessive *a* factors for resistance of the Ambalema type. All of the backcross plants were partially sterile, indicating their hybrid origin.

DISCUSSION AND CONCLUSIONS

The Ambalema type mosaic-resistant burley tobacco evidently does not contain any factors for mosaic susceptibility, as a single *A* factor for susceptibility has been demonstrated to be dominant over three *a* factors for resistance. *Nicotiana glutinosa* contains the *N* or necrotic spotting factor but when grown at 97° F. develops typical mosaic mottling. Therefore, the results of the present paper may be interpreted as proving that *N. glutinosa* contains not only the dominant *N* factor for necrotic spotting but also a dominant factor for susceptibility to mosaic. The temperature studies of McKinney and Clayton may be interpreted as showing that a temperature of 97° F. makes the *N* factor in *N. glutinosa* temporarily nonfunctional but allows the susceptibility factor to operate, with the result that *N. glutinosa* develops typical mosaic mottling. Mosaic-resistant tobacco varieties containing the *N* factor are essentially mosaic-susceptible varieties with the *N* or necrotic spotting factor probably substituted for the N' or n'[6] factor of *N. tabacum* but otherwise the genetic make-up seems to be identical with that of ordinary susceptible varieties of *N. tabacum*.

A variety of the genetic make-up NN a_1a_1 a_2a_2 should be virtually immune to the tobacco mosaic virus because necrotic spots should develop so slowly that localization would be complete. This combination is still in the process of development.

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MORPHOLOGY AND VARIABILITY OF THE CUCURBIT BLACK ROT FUNGUS [1]

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INTRODUCTION

The cucurbit black rot fungus was first described by Fautrey and Roumeguère (*17*) [3] on a Chinese variety of cucumber in France in 1891 and designated as *Ascochyta cucumis*. A little later in the same year Passerini (*18*) described the fungus *Didymella melonis* on *Cucumis melo* L. in northern Italy without knowledge of its association with *Ascochyta*, while Chester (*2*) described the same fungus on *Citrullus vulgaris* Schrad. in Delaware under the name *Phyllosticta citrullina* without recognizing the ascigerous stage. After further study in Delaware, C. O. Smith (*19*) (1905) named it *Ascochyta citrullina*. When he discovered the ascigerous stage, he described it as *Sphaerella citrullina*. Grossenbacher (*4*) designated the imperfect stage of the fungus a member of the genus *Diplodina*, and referred the ascigerous stage to the genus *Mycosphaerella*. Potebnia (*14*) reported a fungus on *Cucumis melo* L. under the name *Ascochyta melonis* which was considered to be closely associated with *Didymella melonis* Pass. He was of the opinion that *A. citrullina* (Chester) C. O. Sm. on watermelon was a form intermediate between *A. cucumis* Fautr. and Roum. and *A. melonis* Poteb. As a result of further studies on Fautrey's collection, Keissler (*10*) considered the three names to be synonyms, and recognized *Ascochyta cucumis* Fautr. and Roum. as the first valid name for the imperfect stage. It is interesting to note that Ferraris (*3*) published a species on *Cucumis melo* L. in the northern part of Italy under the name of *Sphaerella melonis* and claimed that the asci are nonparaphysate. The description of Ferraris' *Sphaerella melonis* showed that it is the same fungus as Passerini's *Didymella melonis* and of C. O. Smith's *Sphaerella citrullina*. There is no specific difference between either one of the European forms and the American fungus.

The pleomorphism and variability of this fungus were the cause of some of the confusion in its early classification of the imperfect stage. Chester (*2*) described it as *Phyllosticta citrullina* on account of its continuous spores, while Keissler (*10*), in studying Fautrey's collection on *Cucumis melo* L., failed to recognize the micropycnospore

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[3] Italic numbers in parentheses refer to Literature Cited, p. 101.

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stage as a phase of the fungus, and thought it to be *Phyllosticta orbicularis* Ell. and Ev. More recently, Wiant (*24*) pointed out that there is considerable variation in the size of pycnidia and perithecia and in the relative abundance of nonseptate spores. He also observed that the extent of sporulation varied in different isolates.

The present study is concerned with the variability of the morphology and cultural characters of the cucurbit black rot fungus. It is shown that the proper binomial of the fungus is *Mycosphaerella cucumis* (Fautr. and Roum.) nov. comb.

EXPERIMENTAL RESULTS

SOURCE AND CHARACTERISTICS OF CULTURES

The black rot disease was found to be endemic in a watermelon-growing area near Merrimac, Wis. Vines of the Hawkesbury variety of watermelon were collected on August 27, 1945, and from these tissue-fragment cultures were made. On September 13, 1945, specimens containing both pycnidia and perithecia were obtained from the same source. Single-spore cultures from ascoespores and from pycnospores were made. The isolates from pycnospores were of two types. One type sporulated very sparsely; the other produced pycnidia promptly and abundantly. The first type was designated as A and the second type as As.

Successive transfers of the aerial mycelium of As isolates resulted in cultures similar to A. On the other hand, As sectors appeared suddenly and rather frequently in A cultures. From mature perithecia in such sectors 54 single ascospores were picked. The colonies which resulted fell into 4 groups. About 8 percent were As type; about 85 percent were of a type designated as $B-a$; about 2 percent were of a type designated as $B-1a$; and 5 percent were referred to as type $B-b$. The cultural characters of the 5 types are described briefly as follows:

A.—Aerial mycelium vigorous, white, becoming gray with age; submerged mycelium dark olive to black; growth fairly rapid; usually sterile; sectors of As type with fruiting bodies appearing frequently.

As.—Aerial and submerged mycelium identical with that of A, aerial mycelium turning gray more rapidly; growth fairly rapid; abundant dark to black pycnidia, perithecia, and "pseudoperithecia."

$B-a$.—Aerial mycelium usually absent, very scanty when present; submerged mycelium hyaline at first, becoming dark olive to black; numerous small pycnidia, light brown at first, turning brownish black; black perithecia and "pseudoperithecia" appearing abundantly later.

$B-1a$.—Aerial mycelium absent or sparse; submerged mycelium hyaline at first, becoming olive green to greenish black; numerous large pycnidia produced promptly; perithecia and "pseudoperithecia" appearing late and sparsely.

$B-b$.—Original character intermediate between $B-a$ and As, later changing to As.

The types mentioned above and certain other types described later are illustrated in figure 1.

PYCNIDIAL STAGE

There is evidence in certain species of *Mycosphaerella* that microconidia function as spermatia. The earliest record of the so-called spermagonium was made by Higgins (*6*) (1914) in *M. nigerristigma* Higgins. Klebahn (*12*) applied the name micropycnidia to the small

conidia in *M. hieracii* (Sacc. and Briosi) Jaap. Snyder (*20*) pointed out that the so-called pycnospores of *M. brassicicola* (Fr.) Lindau did not germinate under the conditions studied and suggested that they probably were spermatia. There is no evidence as yet that micro-pycnospores of *Mycosphaerella* having an *Ascochyta* stage function as spermatia.

Pycnidia bearing either micropycnospores or macropycnospores were present constantly in the sporulating cultures of *Mycosphaerella cucumis* studied. Production of one or both types on certain media varied with the strain of the organism. In general, pycnidia bearing microspores could be induced in *A* cultures by ultraviolet irradiation on potato-dextrose agar, while in *As* cultures they were produced
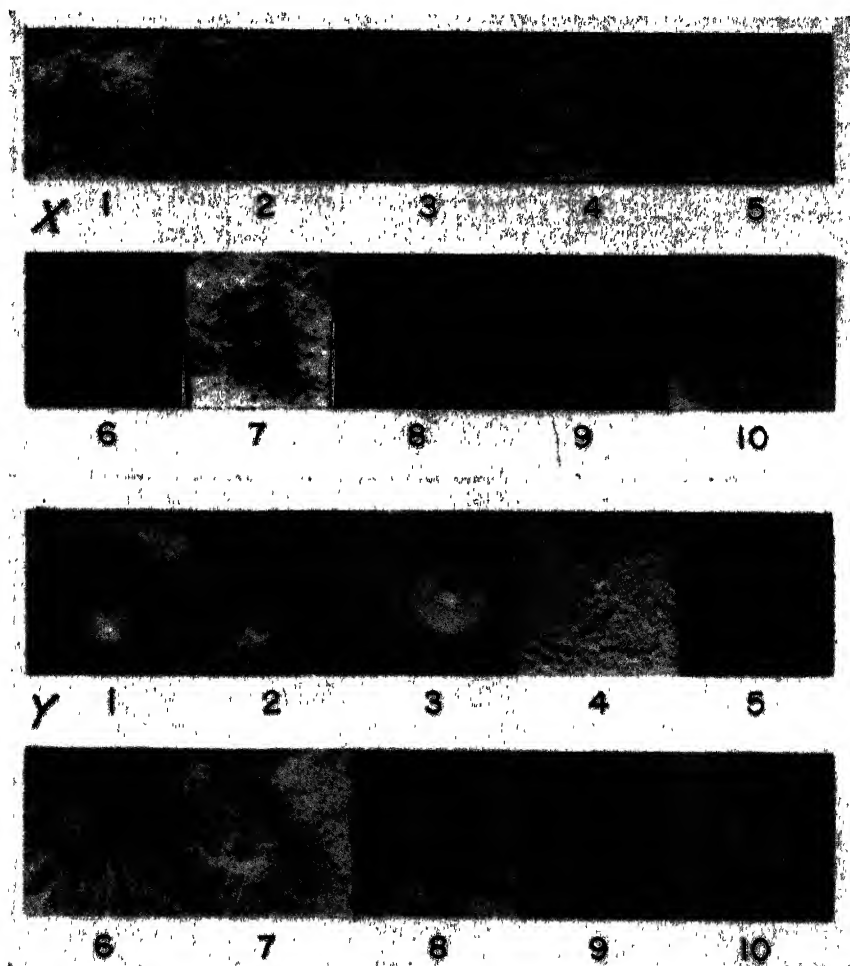
FIGURE 1.—Seven-day cultures of variants of *Mycosphaerella cucumis*. *x*, Growth on oatmeal-dextrose agar. *y*, Growth on potato-dextrose agar. The strains are indicated as follows: *1, As* (wild type); *2, A; 3, A-1; 4, A-2; 5, A-2s; 6, B-a; 7, B-b; 8, B-1a; 9, B-1b; 10, B-2.*

naturally on both potato-dextrose agar and oatmeal-dextrose agar. In *B–a* and *B–1a* cultures both types of pycnidia occurred on oatmeal-dextrose agar, but only pycnidia producing microspores occurred on squash-extract agar. On potato-dextrose agar, microspores predominated in *B–a* cultures and macrospores in *B–1a* cultures. The sizes of pycnidia and pycnospores are compared in table 1. It is evident that pycnidia varied greatly in size on culture media. The range of size of pycnidia from naturally infected watermelon stems was somewhat narrower (50 μ–150 μ). The size and shape of pycnidia from several sources are illustrated in figure 2.

TABLE 1.—*Measurements of pycnidia and pycnospores of variants of Mycosphaerella cucumis*

Measurement	Agar medium	Range and mode of size in variants indicated			
		A	*As*	*B–a*	*B–1a*
		Microns	*Microns*	*Microns*	*Microns*
Diameter of pycnidia	Potato-dextrose.	90–200 [1]	80–180	60–150	60–170.
	Oatmeal-dextrose.	60–200	80–200	60–165	85–330.
	Squash-extract.			70–160	80–180.
Width and length of micropycnospores.	Potato-dextrose.	1.5–3 × 4–8	1.5–3 × 3–9	2–4 × 4–10	
	Oatmeal-dextrose.	2–2.5 × 6–9	1.5–3 × 3–9	2–4.5 × 4–9	2.5–5 × 4–9.
	Squash-extract.			2–3 × 6–8	1.5–2.5 × 5–9.
Width and length of macropycnospores.	Potato-dextrose.				2.5–5 × 5–14.
	Oatmeal-dextrose.			5–7 × 10–13.	5–5.5 × 9–1?

[1] Produced by irradiation with ultraviolet.

Most of the pycnospores obtained from naturally infected material were nonseptate, with a size range of 2.5μ–4.5μ x 5μ–10μ. Variation in septation of pycnospores of all the isolates was considerable on culture media. Pycnospores could be divided as to size into two classes, designated herein as micropycnospores and macropycnospores. The size range of the micropycnospores was quite similar in the variant types and on the different media used. Generally speaking, the micropycnospores were unicellular. The macropycnospores of *B–1a* were mostly uniseptate and occasionally biseptate, while the macropycnospores of *B–a* were mostly nonseptate. In a reisolate of *E–1a* type from overwintering watermelon stems all pycnospores were uniseptate. There was no relation between size of pycnidium and size of spore produced. Some very small pycnidia produced macropycnospores and some large pycnidia produced micropycnospores. A few microspores sometimes appeared in a pycnidium in which macrospores predominated but the reverse was not noted. The small pycnidia of *B–a* on oatmeal-dextrose agar and the larger pycnidia of *B–1a* on the same medium produced mostly macropycnospores.

The micropycnospores and macropycnospores did not appear to differ in function. They were equally germinative and developed
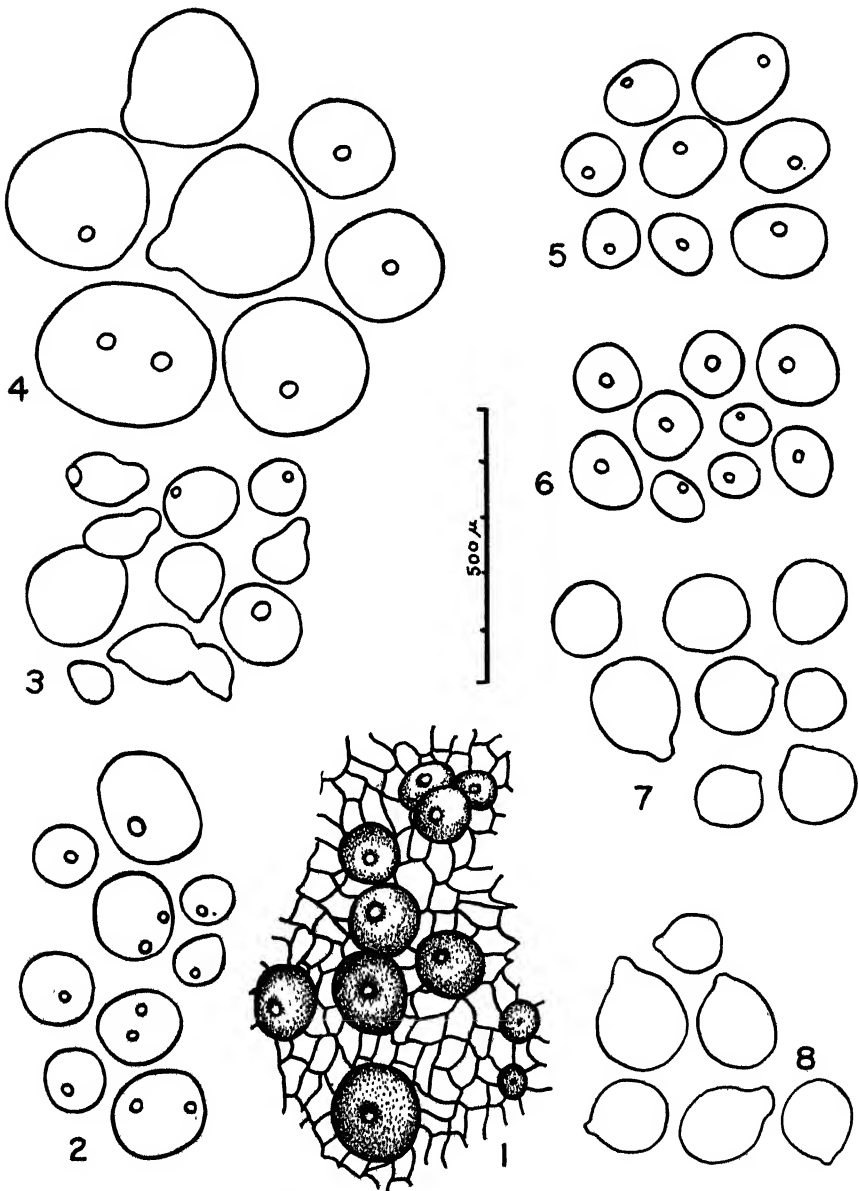
FIGURE 2.—Variation in size and shape of pycnidia. *1, As,* wild type on watermelon stem; *2, As* type on oatmeal-dextrose agar; *3, A* type on potato-dextrose agar after ultraviolet irradiation; *4, B–1a* type on oatmeal-dextrose agar; *5, B–1a* type on squash-extract agar; *6, B–a* type on squash-extract agar; *7, B–a* type on oatmeal-dextrose agar; *8,* "pseudoperithecia" of *B–a* type on oatmeal-dextrose agar.

into colonies true to the type from which they were taken if no mutation occurred. Later studies on the development of perithecia did not indicate that the micropycnospores were concerned with fertilization.

Types of pycnospores are shown in figure 3.

ASCIGEROUS STAGE

Grossenbacher (*4*) studied the perithecia from diseased muskmelon and recorded their diameter as ranging from 100μ to 165μ. Recently Wiant (*24*) observed the perithecia from several curcurbit species and found them to vary in diameter from 71μ to 224μ. The dark-brown
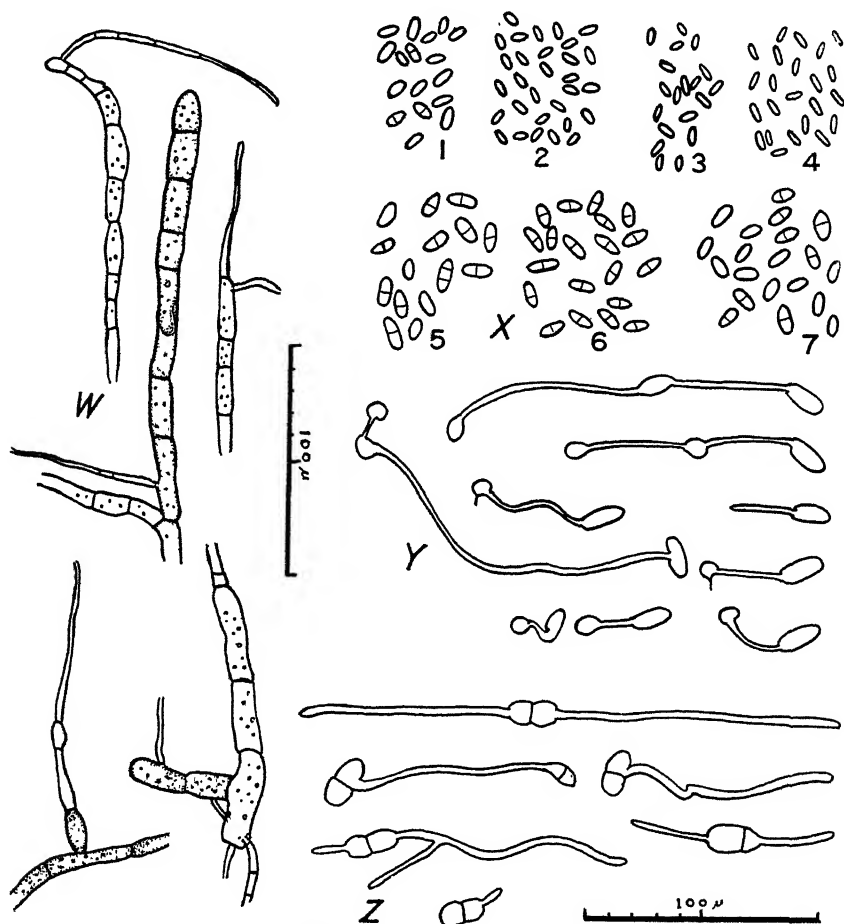
FIGURE 3.—*W*, Old mycelium of *As* type on potato-dextrose agar. *X*, Variation in size, shape, and septation of pycnospores: *1*, from naturally infected watermelon stem; *2*, *As* type on oatmeal-dextrose agar; *3*, *B–a* type on squash-extract agar; *4*, *B–1a* type on squash-extract; *5*, *B–1a* type on oatmeal-dextrose agar; *6*, *B–1a* reisolate after overwintering on watermelon stem; *7*, *B–a* type on oatmeal-dextrose agar. *Y*, Micropycnospores germinating (24 hours at 24° C.) in 2 percent orange extract. *Z*, Macropycnospores germinating (24 hours at 24° C.) in 2 percent orange extract.

to black color and the globose to inverted oval shape have been recorded in all descriptions since Grossenbacher (*4, 13, 24*). In the present investigation the perithecia were mostly globose to subglobose either on naturally infected watermelon stem or on culture media. The mature perithecia were dark brown to black. They were partially embedded or erumpent in the diseased tissue and on culture media. Measurements of perithecia, asci, and ascospores given in table 2 show that the perithecia were largest in *B-a* and *B-1a* cultures. The sizes of asci and ascospores were about the same in all isolates.

TABLE 2.—*Average measurements of perithecia, asci, and ascospores on watermelon and on oatmeal-dextrose agar*

Organ	From water-melon stem canker	From the isolate indicated grown on oatmeal-dextrose agar		
		As	*B-a*	*B-1a*
	Microns	Microns	Microns	Microns
Perithecia	90–120	75–125	78–190	70–230
Asci	6–10×56–70	8–10×55–88	6–9×65–80	8–12×55–80
Ascospores	4. 5–6×8–15	5–7×12–15	6–9×12–15	5–7. 5×11–15. 5

The fascicled asci were hyaline and each ascus contained eight bicelled fusiform ascospores either in one or in two series. Many mature perithecia were studied. No paraphyses were found among the asci. However, in young perithecia there were paraphysislike structures which were close to those illustrated by Klebahn (*13*) for *Didymella lycopersici*. Perithecia of different ages were picked and studied. It was found that in certain young perithecia such paraphysislike structures were more numerous than asci while in older ones they were less common or absent. Since the asci did not all mature at the same time and since abortion was common, these structures were regarded as immature or abortive asci and not as true paraphyses.

A peritheciumlike fruiting body was always found associated with the perithecia in agar cultures, usually being produced slightly earlier than the perithecia. Such structures were indistinguishable from the true perithecia in shape, color, and size (110μ–160μ). Instead of asci and ascospores, there were produced within, spores in chains which separated later. Spores were of one to three, mostly two cells, hyaline, nearly spherical, and 6μ×7μ to 6μ ×19μ in size. They were discharged from the fruiting body along with granular material. Since the internal structure of the fruiting body was distinct from that of the perithecium, the name pseudoperithecium was applied.

Various phases of the ascigerous and pseudoperithecial stages are illustrated in figure 4.

DEVELOPMENT OF FRUITING BODIES

The developmental history of the fruiting bodies has been studied in other species of *Mycosphaerella* (*6, 7, 8, 25*), but the details of the development of perithecia of *M. cucumis* have not been reported previously. Study of several hundred monosporic isolates revealed that this fungus was strictly homothallic. The prompt development
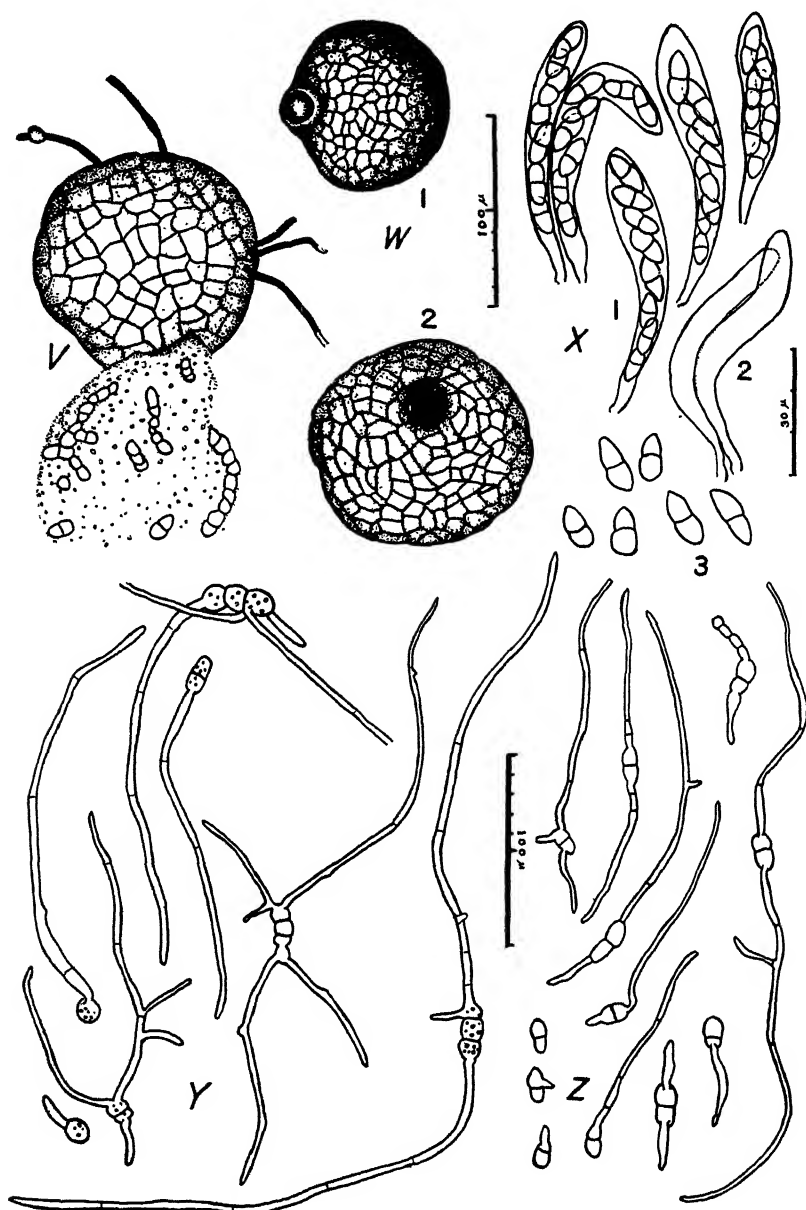
FIGURE 4.—*V*, Pseudoperithecium discharging granular contents and pseudoasco-spores. *W*, *1*, *2*, Mature perithecia. *X*, *1*, Mature asci with ascospores. *Y*, Pseudoascospores germinating (24 hours at 24° C.) in 2 percent orange extract. *Z*, Ascospores germinating in the same medium.

of pycnidia and perithecia in all fertile cultures facilitated the present studies. Observations were made on the cultures seeded at one side of oatmeal-dextrose agar plates. A young *B–a* culture was used because all three types of fruiting bodies were produced, aerial mycelium was absent, and the submerged mycelium grew relatively slowly. Sketches were made with the aid of a camera lucida.

Formation of pycnidia was initiated by the looping of the end of a main hypha or of its branch (fig. 5). As a rule, septation of the looping hypha increased as the size and number of coils increased. By the time three to five loops had formed, numerous slender hyphae with dense cytoplasm were developing from the inner surface of the entangled loops. These slender hyphae were possibly pycnosporal primordia which later developed into pycnospores. When more loops developed and a young pycnidium took form, the hyaline mycelium changed to yellowish brown. As maturity was approached, the color turned darker. The loops of a pycnidium in certain cases were contributed by different hyphae from the same thallus. One characteristic of the mycelium which was destined to form pycnidial loops was the presence of many swollen cells in the older part of the thallus. Such cells were elliptical or oval in shape and had very thin walls. The contents appeared to be very dilute and a few seconds' exposure outside the culture dish resulted in their immediate collapse. The early stage of loop formation was observed in both sporulating and nonsporulating isolates. In the latter the development ceased at the formation of loops and without the formation of pycnosporal primordia.

The first step in the development of perithecia was the formation of a terminal ascogonium that was nonseptate and had very dense cytoplasm (fig. 5). The ascogonium-bearing hypha was either straight or coiled. The antheridial hypha originating from a neighboring thick hypha of the same thallus was long and slender with granular, usually hyaline, cytoplasm. As the antheridial hypha made contact with the ascogonium, the latter began to become septate and coil around the terminal swelling of the former. The behavior of the nuclei was not studied. After intensive coiling and septation of the cells below the terminal cell, a rudimentary perithecium began to take form. The color of the external cells then turned olive brown. Accessory hyphae were sometimes observed. Because of the slenderness of these accessory hyphae, they were suspected of being trichogynes characteristic of certain other species of *Mycosphaerella*, but they were not visible at the time of contact of antheridium and ascogonium. Mature perithecia were finally observed, presumably after fertilization of the ascogonia.

In culture, the ascogonium sometimes failed to make contact with the antheridial hypha. In such cases, the mature "perithecia" failed to produce any asci and ascospores and, instead, chains of ascosporelike structures were formed. Further study would be necessary to establish whether or not there is a positive correlation between failure of the initial to develop into a perithecium and lack of fertilization. The chains eventually separated into sporelike bodies, each
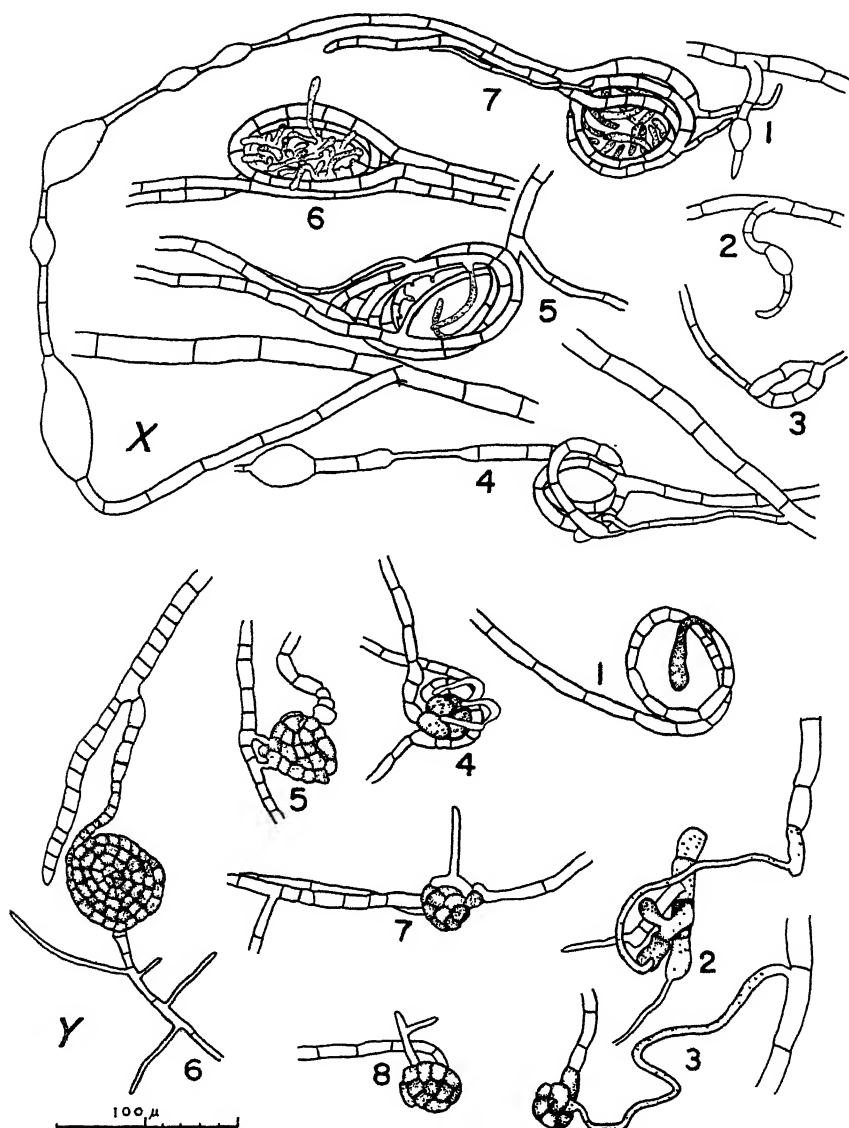
FIGURE 5.—Development of sexual and asexual fruiting bodies in a *B–a* culture on oatmeal-dextrose agar. *X*, Formation of pycnidium: *1, 2,* fertile hyphae; *3,* early stage of loop formation; *4–7,* stages in development of pycnosporal primordia. *Y*, Formation of perithecium and pseudoperithecium: *1,* coiling ascogonium; *2,* antheridial hypha making contact with an ascogonium; *3,* swelling tip of the antheridial hypha with the ascogonium coiling around it; *4, 5, 6,* postfertilization stages; *7, 8,* nonfertilized ascogonia developing into pseudoperithecia.

consisting of one to three cells. The cells were roughly spherical and the content was not so dense as that of ascospores. In reality these structures were homologous with pycnidia and pycnospores. In this paper the terms "pseudoperithecium" and "pseudoascospore" are applied to them.

SPORE GERMINATION

Pycnospores, ascospores, and pseudoascospores germinated readily in hanging drops of 2 percent orange extract (figs. 3, 4). Germination was very poor in sterile tap water.

The first step in the germination of pycnospores was the swelling of the cell to several times its original size. The second step was the formation of a hyaline germ tube. In the case of micropycnospores, a globular cell was usually formed at the tip of the germ tube, which either sent out a slender thread perpendicular to the axis of the germ tube or continued to grow and form another globular structure at the tip. In the case of macropycnospores, each cell of the bicelled spore sent out a germ tube which either remained nonseptate and nonbranched or became septate and branched immediately. Branching occurred at any point along the germtube. Secondary sporelike structures were sometimes found on the terminals of side branches similar to those observed by Grossenbacher (*4*). The ascospores and pseudoascospores germinated in the same manner as pycnospores except that there was no secondary sporelike structure at the tip of hyphae.

VARIABILITY OF CULTURAL CHARACTERS

In his recent studies of the black rot fungus, Wiant (*24*) reported isolates having various sporulating capacities and different cultural characters. In this investigation spores and hyphae were stained with 1 percent crystal violet for 1 minute, and the surplus stain was removed with distilled water and blotting paper. The preparation was cleared with 1 percent picric acid and then with clove oil. The cells of ascospores and pycnospores were shown to be uninucleate and those of the mycelium multinucleate. Since the fungus was homothallic and since individual thalli arose from uninucleate cells of spores, the nature of variability of the fungus was of particular interest.

MONOSPORIC ISOLATIONS

The sporulating strains, *As*, *B–a*, and *B–1a*, were grown on oatmeal dextrose agar because of the profuse and early production of fruiting bodies. In general, pycnospores were picked from 7-day cultures and ascospores from 15-day cultures. The method was that used by Keitt and Langford (*11*), but no attempt was made to isolate the ascospores in their natural order.

A very dilute suspension of pycnospores was smeared over the surface of a potato-dextrose agar plate with a sterilized glass spatula. This seeded plate was incubated overnight at 24° C. When the germ tubes were beginning to protrude, spores were picked with a specially prepared glass needle under a binocular microscope. Only distinctly germinating spores were transplanted on oatmeal-dextrose agar plates. Six spores were usually arranged in a circle on one plate.

The incubation temperature was maintained at 24°. The cultural characters of the isolates were recorded and compared at the end of 1 week. Those with the same characters were grouped and given the same number.

The first picking of 100 ascospores from the *As* strain yielded 99 *B–a* colonies and 1 *As* colony. The single *As* colony was transferred to oatmeal-dextrose agar and from it 112 ascospores were picked, of which 96.4 percent produced *As* colonies; 1.8 percent produced *A* colonies; 1.8 percent were of a new type, designated as *A–1*, which was a sterile albino with aerial mycelium similar to that of *A* and *As*. Each of 224 pycnospores picked from the original *As* strain yielded an *As* colony. Variants appeared to arise only from ascospores in this set of experiments, but the lack of variants from pycnospores may have been due to the relatively small number observed.

From 108 ascospores from strain *B–a*, only *B–a* colonies developed. From 80 ascospores from a *B–a* culture derived from an ascospore in the first picking only *B–a* colonies developed. About 20 percent of the *B–a* colonies were lighter in color than the rest. They were designated as *B–a(1)* and the others as *B–a(d)*. From 104 pycnospores isolated from a *B–a(1)* colony, 95 percent yielded *B–a(1)* and 5 percent *B–a(d)* colonies. From 112 pycnospores selected from a *B–a(1)* colony of the previous picking, 98 percent of the colonies were *B–a(1)* and 2 percent were *B–a(d)*. It appeared that *B–a(1)* was a mutant from *B–a(d)* and tended to throw frequent *B–a(d)* mutants.

From 140 ascospores from a *B–1a* culture all colonies were *B–1a*. In 3 successive monopycnosporic isolations covering a total of 416 colonies, all were true *B–1a* type.

Since the *B-b* culture in its early stage of growth showed a mosaic of characters of *B–a* type and *As* type and later developed *As* characters entirely, it was thought that possibly it came from an *As* ascospore but that a *B–a* mutation occurred in one of its young hyphae, and the mutant character was overrun by the rapid growth of the *As* of the mycelium. This supposition was shown to be true by a study of monoascosporic isolations from *B-b* type. From 75 monosporic colonies, 23 percent were *As* and 77 percent were *B–a*. No homogeneous *B-b* type cultures were secured by monoascosporic isolations from *As* or *B–a* type.

A diagrammatic summary of the type of culture secured by a study of single-spore lines follows:
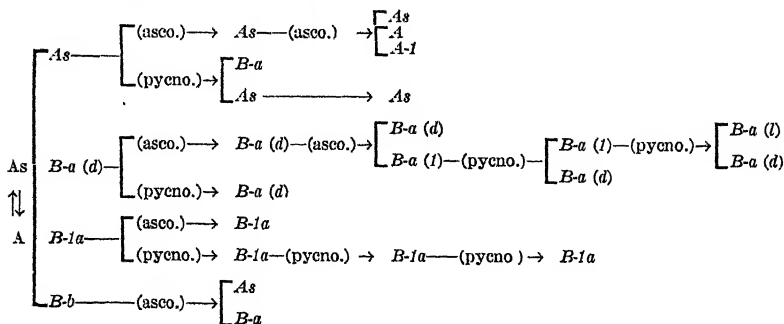
In order to observe a comparatively large population during a brief period, the dilution-plate method was used. Although this method had the disadvantage that two or more sporalings might grow together to form a single colony, the population that could be observed for variation was much greater than when individual spores were picked. Monosporic *As* and *B-1a* cultures were used in this test. The spore suspension of each culture was so adjusted that 1 milliliter of sterile water contained approximately 50 pycnospores of *B-1a* or 25 spores of *As*. One milliliter of the prepared spore suspension was pipetted aseptically into the oatmeal-dextrose agar plates and was made to spread evenly over the surface. Twenty plates of *As* and 30 plates of *B-1a* were made. All plates were incubated at 24° C., for 7 days in the case of *B-1a* and for 15 days in the case of *As*. The total number of colonies established in each plate was counted, and any colony that was slightly different from the parent culture in color and growth was recorded and isolated for further comparison both by mass transfer and by isolation of monopycnospores.

The total of resulting colonies from *As* was 371, of which 3 percent were *A* type, 1 percent were *B-1b* type, and 96 percent were *As* type. *B-1b* type was very similar to *B-1a* type, except that it assumed a deep olive-green color at an early stage of development. The difference between *B-1a* and *B-1b* types corresponded exactly to that between *B-a* (1) and *B-a* (d) types. Some monopycnosporic isolates of *B-1b* were uniform for type characters. Dilution plates of a *B-1a* culture yielded a total of 1,585 colonies, of which 0.4 percent was *B-1b* type and 99.6 percent *B-1a* type. A pycnospore suspension of a *B-1b* culture was prepared as in the case of *B-1a* and 100 dilution plates were made. Each of 5,126 colonies which developed was *B-1b* type. From this study it was evident that in monosporic cultures of *As* the type and number of mutants was greater than in *B-1a* cultures. The difference in color of *B-1a* and *B-1b* at the early stage of development was slight, and it disappeared as the colonies grew. The results with the dilution-plate method were similar to those obtained from monosporic isolations.

ISOLATION OF MYCELIAL CELLS

The frequent occurrence of *As* sectors and the sudden appearance of *As* fruiting bodies in *A* cultures led to consideration of the variability arising from nuclear changes in mycelial cells. Since the *A* culture was obtained from early monosporic isolations, the purity of the culture was beyond question. The problem to be solved was the uniformity in the genotype of the cells of the mycelium that might be detected by phenotype analysis. In order to render such study feasible, the isolation of mycelial cells at random was carried out.

Two *A* cultures from monopycnosporic isolation from the natural host substrate were available. One was 1 year old, the other was 1 month old. The mycelium of these cultures was removed and washed several times with sterile water. The washed mycelium from one plate culture of a given isolate was minced aseptically for 15 minutes in a Waring Blender. To 1 ml. of the resulting mycelial suspension was added 9 ml. of sterile water. Then with the aid of a

sterile glass spatula, one drop of the suspension was smeared over a potato-dextrose agar plate. The technique for picking spores was again used in the picking of mycelial cells. Only those pieces of hyphae which consisted of two to three cells were picked and transplanted to oatmeal-dextrose agar plates. Plates were incubated at 24° C. and records were taken at the end of the seventh day when fruiting bodies were appearing on certain colonies.

In the case of the 1-year *A* culture, a total of 96 pickings was made. Only 24 percent of these pickings developed into colonies. Among the developing colonies, 87 percent were *As* type and 13 percent *A* type. A total of 53 pickings was made from the 1-month *A* culture. All of them developed into colonies, of which 68 percent were *As* type and 32 percent *A* type. The variation of the *A* type culture to the *As* type and vice versa occurred naturally and spontaneously. It was not possible to separate these 2 types and propagate them in their own characters indefinitely.

RELATION OF MEDIUM TO GROWTH AND SPORULATION

In an attempt to induce sporulation of the fungus, Wiant (*24*) employed a number of media including corn-meal agar, oatmeal agar, and cucurbit stems. Sporulation was scant in each of these, only a few fruiting bodies appearing occasionally. In the studies just reported sporulation was governed by genetic factors. However, it was of interest to determine whether sporulation in the sterile type *A* could be induced by the nutrient. The agar media used are listed in table 3. Dextrose and peptone were used at the rate of 10 grams per liter. Other ingredients were used at rates indicated elsewhere (*16*).

TABLE 3.—*The effect of various agar media on sporulation of an A type culture of Mycosphaerella cucumis*

Medium	Average growth rate	Color of the substratal mycelium	Aerial mycelium [2]	Sporulation at 144 hours
	Mm. per hour			
Potato	0 53	Pale olive	+	None.
Potato-dextrose	.91	Dark greenish black	+++	Do.
Potato-peptone	.84	Salmon	++	A few.
Corn meal	1.75	Pale olive	+	None.
Corn meal-dextrose	.72	Greenish black	++	A few.[3]
Corn meal-peptone	.79	Salmon to brownish	+++	Sparse.
Czapek's solution	1.96	Colorless	−	None.
Czapek's solution-dextrose	.79	Dark olive	+++	Sparse.
Czapek's solution-peptone	.53	Salmon	+++	None.
Czapek's solution dextrose-peptone	.86	Yellowish brown	++++	Sparse.
Bean	.86	Brownish olive	−	None.
Bean-dextrose	.97	Dark olive green	+++	Sparse.[3]
Bean-peptone	.67	Purplish	+++	None.
Bean-dextrose-peptone	.79	Brownish	+++	Sparse.[3]
Oat-paste	.92	Dark greenish black	+++	Abundant.[3]
Oat-paste-dextrose	.79	Dark greenish black	+++	Do.[3]
Oat-paste-peptone	.79	Pale yellowish brown	+++	None.
Oat-paste-peptone-dextrose	.92	Brown	++++	Abundant.
None	1.57	Colorless	−	None.
Dextrose	.82	Pale olive	−	Do.
Peptone	.39	Salmon	+++	Do.
Dextrose-peptone	.90	Brownish	++++	Do.

[1] Mycelium was weak and sparse.
[2] −=none; + to ++++ indicate increasing amounts of aerial mycelium
[3] Primordial and mature perithecia also occurred

An *A* culture from a monopycnosporic transfer from the *As* type was used.   Five plates of each culture medium were incubated at 24° C. for 144 hours.   Sporulation, rate of growth, color of the submerged mycelium, and extent of aerial mycelium were recorded.

The results in table 3 show that the growth was most rapid when dextrose was present.   In a synthetic medium, such as Czapek's solution, best growth occurred with dextrose and peptone.   The peptone-dextrose effect was less significant in media rich in organic materials.   The presence of dextrose induced olive to greenish-black color while peptone induced salmon to purplish-brown color of sub-merged mycelium.   Peptone usually induced more vigorous aerial mycelium than dextrose, but the most abundant development occurred when both were present.

Sporulation was relatively abundant only on oat-paste agar and when dextrose alone was added to the latter, but it was prevented when peptone alone was added.   When dextrose was added with peptone the inhibitive effect of the latter was prevented.   Sparse sporulation occurred on bean-peptone dextrose and on corn meal-peptone agar; it was rare on corn meal-dextrose and potato-peptone agars.   When sporulation occurred, the cultural characters were very similar to those of *As* cultures.   In earlier experiments it was shown that pycnidial loops occurred in all sterile cultures but devel-opment usually ceased before sporulation.   It is possible, therefore, that the presence of certain nutrients or growth factors in the media mentioned enabled the *A* mutant to become as fertile as the wild type, *As*.   Evidence has already been presented that the *As* type might exist in *A* cultures without expressing itself; it is possible, therefore, that the oat-paste medium promoted the growth and ex-pression of the *As* type which was already present.   Nevertheless, when the above media were used for culture of other sterile strains (*A-1*, *A-2*, and *B-3*) no sporulation was ever observed, although pycnidial loops were common.   This difference may be due to a difference in the nutritional requirements of mutants.

EFFECT OF IRRADIATION ON SPORULATION

The effect of irradiation on the sporulation of fungi has been re-ported by Stevens (*21, 22, 23*) and by Ramsey and Bailey (*15*).   In the present investigation 7-day *A* cultures on potato-dextrose agar plates, showing vigorous aerial mycelium, were irradiated with a mercury-quartz lamp (Westinghouse Sterilamp).   The plates were placed 6 inches below the source of light with covers removed.   The intervals of treatment were ½, 1, 5, 10, 15, 20, 30, and 40 minutes. After the treatment, all plates were kept in complete darkness at 24° C. The nonirradiated plates served as controls.   On the fourth day after irradiation, sporulation occurred in plates irradiated 1 minute to 40 minutes, being most abundant in the 15-, 20-, and 30-minute treatments.   None of the control plates showed a trace of fruiting bodies.   The aerial mycelium of the irradiated cultures had all turned gray and collapsed while abundant black pycnidia appeared amongst the collapsing mycelium.   The pycnidia were very similar to those of the *As* culture.

A series of experiments was then carried out on the media listed in table 3. In addition to sporulation on oat-paste agar, as shown earlier to occur without irradiation, the amount of sporulation was increased on those media in which it occurred only sparsely without irradiation. The most significant effect, however, was on potato-dextrose agar on which no sporulation occurred normally. A slight amount of sporulation appeared on corn-meal agar. The relative number of perithecial primordia remained constant on oat-paste agar, oat-paste-dextrose agar, and bean-dextrose agar in both irradiated and nonirradiated plates, but the maturity of the perithecia was hastened and the proportion of mature perithecia to pseudoperithecia increased with irradiation. When potato-dextrose agar plates were irradiated before they were seeded, no sporulation occurred in the subsequent culture. When *As* cultures were irradiated, the amount of sporulation was not affected.

INDUCTION OF MUTATION BY IRRADIATION

Since spontaneous mutation was observed frequently in the *A* and *As* cultures, the possibility of increasing the frequency and direction of mutation by irradiation with ultraviolet rays was considered. The mercury-vapor type of lamp (General Electric AH–6,100 watts, and 500 volts alternating current) was employed. The wave length was 2,534 Å. and the constant temperature in operation was 25° C. A pycnospore suspension from an *As* culture at a density of approximately 7,500 spores per milliliter of sterile distilled water was used. Ten milliliters of the spore suspension was pipetted into the radiation cell. Before irradiation, 0.2 milliliter was drawn out from the cell and diluted in 100 milliliters of sterile distilled water as a control. After starting the irradiation, 0.2 milliliter of the spore suspension was drawn out and diluted at 10-minute intervals up to 80 minutes. From each dilution of these treatments 10 plates were prepared with 1 milliliter of suspension. All plates were incubated at 24° and records were taken at the end of 6 days. Special attention was given to types of colonies that had not been observed previously.

The percentage of viable spores yielding *A*, *As*, or *B-b* cultures decreased rapidly as the duration of irradiation increased. The mutant, *A–1*, previously observed in monosporic isolations, appeared regularly throughout the treatments. *B–1b* type, which had been isolated by the dilution-plate method from *B–1a* cultures, appeared at 10-minute radiation. Three mutants (*A–2*, *A–2s*, and *B–3*) which had not been observed before, were found. *A–2* appeared first after 10 minutes' irradiation but was more frequent after 30 to 60 minutes. *A–2s* appeared only once and then after 70 minutes' irradiation, while *B–3* appeared at between 40 and 50 minutes. *A–2* and *A–2s* were quite similar; the former was a sterile albino, the latter sporulated and was white at first, turning brown with age. The pycnidia produced in *A–2s* were peculiar in type, since they were usually grouped together to form sclerotiumlike bodies in which several pycnidia were united at the base but were distinct otherwise and had distinct ostioles. The black sclerotiumlike bodies were 0.5–3 millimeter in diameter. The mass of pycnospores discharged at the ostioles was whitish instead of the usual grayish salmon. Type *B–3* grew very

slowly and had thick velvety to woolly aerial mycelium. Both aerial and submerged mycelia assumed a purplish-brown color. No mature fruiting bodies were observed, but microscopic study revealed that the loop formation characteristic of the early stage of normal pycnidial development was present. Nevertheless its development stopped before the formation of a pycnidial wall. Colonies of the mutants described above are illustrated in figure 1.

As shown in figure 6, the percentage of killing caused by irradiation increased rapidly up to 20 minutes. The differences in the percentage of killing at intervals above 20 minutes were not significant. The percentage of mutants in the surviving spores increased rapidly up to 40 minutes' exposure beyond which the rate slowed down. The dif-
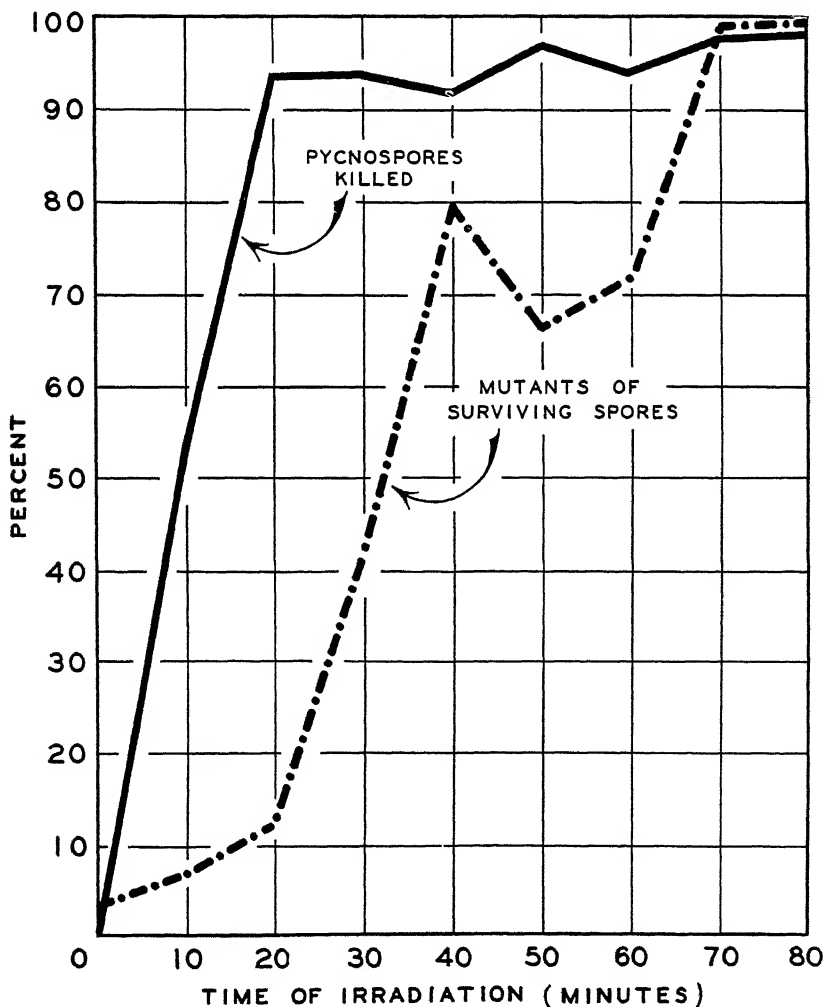
FIGURE 6.—Effect of irradiation on killing and on mutation of pycnospores of *Mycosphaerella cucumis.*

ference in percentage of mutation at 70 to 80 minutes and 40 to 60 minutes is probably not significant since the population of spores which survived the two longest treatments was very small.

INDUCTION OF MUTATION BY HIGH TEMPERATURE

It was observed during very warm summer periods that A sectors appeared more frequently than usual in monosporic isolates from As cultures. Experiments were conducted, therefore, to determine whether temperature influenced the rate of mutation. Two young monosporic cultures were incubated at 24° C. for about 10 days, when fruiting bodies were well formed. Then 1 of the plates was removed to an incubator at 36° C., the other to an incubator at 16°. At the end of 1 week, 10 dilution plates for each treatment were made. All these plates were again incubated at 24° for 1 week, and then the number and type of colonies were recorded. The data in table 4 show that the frequency of A mutations in As cultures was greatly increased after high temperature treatment. The B–$1a$ mutant also occurred after the high temperature treatment. These results, incidentally, offered an explanation of the fact that difficulty was commonly encountered in maintaining the sporulating character of the As cultures in the laboratory during warm summer periods.

TABLE 4.—*The effect of temperature on mutation as shown by the percentage of colonies in type classes*

Treatment °C.	Total colonies	Colonies in type classes—			
		As	A	B–a	B–$1a$
	Number	*Percent*	*Percent*	*Percent*	*Percent*
16	239	98.3	1 7	0	0
36	178	39.4	59.7	0	1.6

TAXONOMIC POSITION OF THE FUNGUS

The present study shows that the imperfect stage of the fungus is extremely variable in size of pycnidia and in size and septation of pycnospores. Moreover, considerable variation in these characters occurs as a result of genetic mutation and the segregation of mutants in culture. This accounts for the confusion in the taxonomic position of the fungus cited in the introduction of the paper. Since Fautrey and Roumeguére (*17*) published the name of the imperfect stage, *Ascochyta cucumis*, in 1891, slightly earlier than that of the perfect stage by Passerini (*18*) in the same year, the first specific name has priority. Therefore the proper binomial of the fungus should be *Mycosphaerella cucumis* (Fautr. and Roum.) nov. comb. The synonymy follows:

Ascochyta cucumis Fautr. and Roum., 1891.
Didymella melonis Pass., 1891.
Phyllosticta citrullina Chester, 1891.
Ascochyta citrullina (Chester) C. O. Sm., 1905.
Sphaerella citrullina (Chester) C. O. Sm., 1905.
Diplodina citrullina (C. O. Sm.) Gross., 1909.
Mycosphaerella citrullina (C. O. Sm.) Gross., 1909.
Ascochyta melonis Potebnia, 1910.
Sphaerella melonis Ferraris, 1912.

DISCUSSION

Although Fautrey and Roumeguére (17) recognized the cucurbit black rot fungus on a variety of Chinese cucumber to be a species of *Ascochyta* and described it under the name *Ascochyta cucumis* in France as early as 1891, the pleomorphism of this fungus caused some confusion in its classification. The small continuous micropycnospores which predominate in nature on the host apparently led Chester (*2*) to describe the fungus as *Phyllosticta citrullina* on watermelon and led Keissler (*10*) to report *Phyllosticta orbicularia* Ell. et Ev. on *Cucumis melo* L. Grossenbacher (*4*) stated that Chester's specimen of *Phyllosticta* might not represent the same pathogen as that described later by C. O. Smith as *Ascochyta citrullina*. The explanation of this discrepancy is found in the fact that this organism may at times produce pycnospores which conform to the genus *Phyllosticta*. Inclusion of the perfect stage of *Ascochyta* in *Didymella* is questionable, even though Passerini (*18*) and Klebahn (*13*) claimed that paraphyses were present. Ferraris (*3*) and American workers (*4, 19, 24,*) did not see any paraphyses among the asci of this fungus. The present observations show that no paraphyses are present in the perithecia but that immature or abortive asci are often present among the mature asci. The abortive asci might have been interpreted erroneously as paraphyses. In view of the priority of *Ascochyta cucumis* Fautr. and Roum., the name of the perfect stage is designated herein as *Mycosphaerella cucumis* (Fautr. and Roum.) nov. comb.

The production of micropycnospores and macropycnospores varied with the strain and the media. Thalli from micropycnospores gave rise to colonies with mature perithecia. In spite of evidence obtained in other species of *Mycosphaerella* that micropycnospores function as spermatia, it is believed that they function as conidia in the black rot fungus.

Homothallism of this genus was first noted by Jones (*9*) and later proved by Hare and Walker in *Mycosphaerella pinodes* (Berk. and Blox.) Stone (*5*). The present pathogen also is homothallic. Fertilization of the ascogonium is accomplished by an antheridium originating from a neighboring hypha of the same mycelium.

Cells of both ascospores and pycnospores were shown to be uninucleate, while those of the mycelium were multinucleate. Wiant's culture from Hubbard squash conforms to the *As* type described herein; his sector of the squash isolate is similar to the *B–1a* and *B–1b* types; his isolate from Puerto Rican cucumber is similar to the *B–a* type. These variants, according to the present studies, originate from the *As* wild type. Wiant also observed the sterile *A* type but failed to point out that *As* and *A* types are spontaneously and reversibly mutable.

The mutability of the *As* strain is high and the direction of mutation is various. The frequency of mutation may be increased by high temperature and by irradiation. That the repeated transferring of mycelium of species of *Ascochyta* increased the sterility of the subsequent cultures has been noted by many workers, including Yu (*26*). This was also observed in the *As* strain. A possible explanation is that the repeated picking of mycelium may greatly increase the probability of purifying the *A* mutant from the *As* strain, since most *As*

mycelium is producing spores and grows less rapidly than that of the mycelial mutant.

Wiant failed to induce sporulation of the culture by the use of different media (*24*). The sporulation of this fungus seems to be controlled by both genetic and nutrient factors as in the case of *Neurospora* (*1*). The loss of the property of synthesizing certain organic substances necessary for sporulation may be responsible for some of the sterility, and may explain the inconsistency in sporulation of the usually sterile *A* type. By using fertile strains, such as *As* and *B-la*, sporulation was readily obtained on all media that Wiant had tried without success.

It is interesting to note that the sterile *A* strain can be induced to sporulate by irradiation of a potato-dextrose culture for 1 to 40 minutes under a mercury-quartz lamp. Ramsey and Bailey (*15*) were able to induce the sporulation of *Fusarium cepae* (Hanzawa) Link and Bailey and *Macrosporium tomato* Cooke by irradiation, and they were of the opinion that it was not due to inhibition but rather to stimulation. Since in the present work the fruiting bodies are exclusively of the *As* type and since *A* and *As* are reversibly mutable, it remains to be determined whether or not irradiation increased the rate of mutation of *A* type to *As* type.

SUMMARY

The perithecia, pycnidia, and pseudoperithecia of *Mycosphaerella cucumis* as well as the spores produced by them, are described. The perithecia varied in size within a much narrower range than the pycnidia. Two types of pycnidia are described. One produced small continuous spores (micropycnospores); the other produced larger spores (macropycnospores) which were continuous, one-septate or rarely two-septate. The types of pycnidia were indistinguishable morphologically except for the size and septation of the pycnospores produced.

Ascospores and pycnospores were uninucleate. Monosporic lines were homothallic. The developmental histories of the sexual and asexual fruiting bodies are described. There was no evidence that micropycnospores functioned as spermatia.

The wild type, *As*, was extremely variable. It mutated spontaneously to *A*, *A-1*, *B-a*, *B-1a*, and *B-1b*. *A* and *A-1* were sterile, while *B-a*, *B-1a*, and *B-1b* sporulated profusely and produced mycelium sparsely. Mutant *A* tended to mutate back to *As* while *A-1*, *B-a*, and *B-1a* were very stable. *B-1b* was apparently a variant of *B-1a* from which it differed only in the early development of the color of the colony. The growth rate and coloration of the submerged mycelium of the fungus varied with the medium. Dextrose in combination with peptone in any kind of medium favored vigorous mycelial growth. Dextrose was responsible for the olive-green color of the submerged mycelium, while peptone induced a salmon to purplish-brown color.

Sporulation of sterile strain *A* was induced on oat-paste agar and by irradiation of a well-developed culture on potato-dextrose agar medium for 15 minutes.

Irradiation with ultraviolet rays induced a higher frequency of mutation and new mutants. *A-2*, *A-2s*, and *B-3*, were obtained by this means. *A-2* and *B-3* were sterile while *A-2s* produced spores in sclerotiumlike groups of pycnidia.

Exposure to a temperature of 36° C. for 1 week resulted in an increase in the number of mutants.

The confusion in earlier literature regarding the taxonomy and nomenclature of this organism is probably due largely to its pleomorphism. Size, shape, and septation of pycnospores and size of pycnidia are extremely variable. The earlier reports of paraphyses in the perithecium are probably due to erroneous interpretations of immature or abortive asci. According to the information available the proper Latin binomial for the fungus is *Mycosphaerella cucumis* (Fautr. and Roum.) Chiu and J. C. Walker.

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RELATION OF SPECIFIC GRAVITY TO SHRINKAGE AND OF THESE FACTORS TO GROWTH IN YELLOW POPLAR[1]

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INTRODUCTION

In recent years a number of investigations have been made to determine the physical properties of various woods and the effect of rate of growth and of certain conditions of growth on these properties. Relatively little work has been done, however, on native diffuse-porous woods, nor has an intensive analysis of relationships been made.

Yellow poplar (*Liriodendron tulipifera* L.), an important Appalachian hardwood and the species used in this study, has been studied by several researchers. Paul and his coworkers (*4, 5, 6*) [2] have given us much of the information now available on the relation of the rate of growth to the specific gravity of the wood produced. Paul (*4*) reported that prolonged suppression of growth resulted in a lowering of the specific gravity of the wood, whereas improved growth conditions in slow-growth virgin trees resulted in an increase in specific gravity. The wood of young trees (70 to 150 years of age) in old-growth stands was heavier than in the old-growth trees (150 to 300 years) of the same stand (*6*). The slightly lower average specific gravity obtained in the second-growth trees as compared to young trees in old-growth stands was explained by the presence of wide annual rings made up of wood of low specific gravity near the pith in trees that had started growth in rather open stands. Paul and Norton (*6*) reported that there was not a very close relationship between rate of growth (rings per inch) and specific gravity in yellow poplar. Nevertheless, they found the rate of growth to be useful in determining the quality of wood if the history of its development was taken into consideration. No definite correlation was established between specific gravity and factors such as climate, soil, and elevation.

Boomsliter (*1*) and Stern (*7*) have studied the mechanical and physical properties of yellow poplar. Their results are based on one or two trees in each case.

This report is intended to check some of the published data and conclusions; to determine quantitatively certain results that have been reported qualitatively only; to include and correlate properties not hitherto studied, such as shrinkage; and to limit the samples to one area of representative commercial stand and thus eliminate possible differences between areas.

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EXPERIMENTAL PROCEDURE

The samples of yellow poplar were obtained from 23 trees on a 200-acre tract in Preston County, W. Va., located in the north-central part of the State. The area is mountainous with steep slopes. The stand was classified as cove hardwood type; the yellow poplar was in mixture with other hardwoods. The samples were collected while the tract was being logged and represented the trees from near the ridges down to the creek bed, or a range of elevation from 1,750 to 2,150 feet. The site is classed as good (site II). The exposure was south to southeast.

One disk, from 6 to 8 inches thick, was sawed from each tree. In 16 of the trees, the disks were taken just above a 16-foot log. In some cases it was necessary to saw off the butt or make a short log first. In 4 trees the disk was sawed off at 8 feet above the stump, in 2 trees at 10 feet, and in 1 tree, at 12 feet. The age of the trees at the height of the test blocks ranged from 130 to 260 years, except one of 78 years; at stump height, the age ranged from 86 to 273 years. If the disk were not to be cut up immediately, they were placed in the cold room at 40° to 42° F. until used. The annual rings were counted and marked on the cross section. Blocks approximately 1 by 1 by 3 inches were sawed from each disk on the north and south diameter. Care was taken to have the fiber alinement parallel to the long axis of the block. When interlocked grain or fluctuating slope of spiral grain was present, the weighted average of the grain slopes was used. Two parallel sides of the block were at right angles to the annual rings; the other two sides were parallel to the rings.

The dimensions of the blocks were measured by a dial-gauge instrument (fig. 1) especially designed and constructed for this purpose. With this instrument, the place of measurement can be marked by drawing a pencil part of the way round the gauge stem and subsequent measurements of the block can be made at the same place. With this instrument it is possible to measure easily and rapidly and to be sure that the same spot will be used in each measurement. If the block is not absolutely square, the use of the same spot in each measurement is very important. To obtain average values with blocks that are not uniform in ring width, it is better to measure at two or more points across the radial surface. Otherwise, the rings-per-inch value which is indicated at the point of measurement should be used. This caliper instrument reads to 0.001 inch throughout a range of 1 inch and is fully as accurate as a vernier caliper. Accuracy is assured by the sensitivity of the gauge, the easily readable dial, which eliminates the eye strain and guessing required to read a vernier scale, and the constant contact pressure of the gauge on the object for a given thickness.

The green volume of each block was determined by suspending it in a beaker of water of known weight and weighing the volume of water displaced. The blocks were then dried very slowly in an experimental dry kiln. Loss of weight was determined by weighed samples. The drying temperature was from 100° to 110° F. Final drying to a moisture content of zero percent was done in an electric oven at 216° F., again in easy stages of drying. After reaching constant weight, the blocks were cooled in a desiccator containing P_2O_5, weighed,

FIGURE 1.—Adjustable dial-gauge caliper for measuring changes of the dimensions of a block.

remeasured, and placed in the oven for several hours. They were then dipped in hot paraffin; the excess on the surface was removed while molten. If no excess remained, dimension measurements were the same after paraffining as before. The oven-dry volume was determined in the same way as green volume.

In all the data presented, the blocks that averaged less than 2 inches from the pith or were less than 20 years old are not included unless specifically stated. Shrinkage values are based on green dimensions. Specific gravity is based on green volume and oven-dry weight.

EXPERIMENTAL RESULTS

I. RINGS PER INCH AND SPECIFIC GRAVITY

The relation of rings per inch to specific gravity for the 345 blocks from 23 trees is shown in figure 2. It is evident that the specific gravity decreases as rate of growth decreases. However, the rate of growth is not closely associated with specific gravity. Expressed statistically, the total or gross correlation coefficient is −0.483. The square of the correlation coefficient, which is the coefficient of determination, indicates that only 23 percent of the variance in the specific gravity of all the samples could be explained by the variation in the rate of growth. The first equation in table 1 (column 5, line 1) is the appropriate one for the curve shown in figure 2. The graph indicates a somewhat better correlation in the region of slower growth than for the total range of rate of growth. An analysis in which only the samples of 20 or more rings per inch (74 in number) were used gave a correlation coefficient of −0.588. Compared to −0.483, the correlation coefficient for the entire curve, this value indicates an appreciably closer relationship between rate of growth and specific gravity in the wood of slower growth.

The standard error of estimate of Y, specific gravity, as indicated in figure 2, is ±0.027. This is the square root of the mean square of
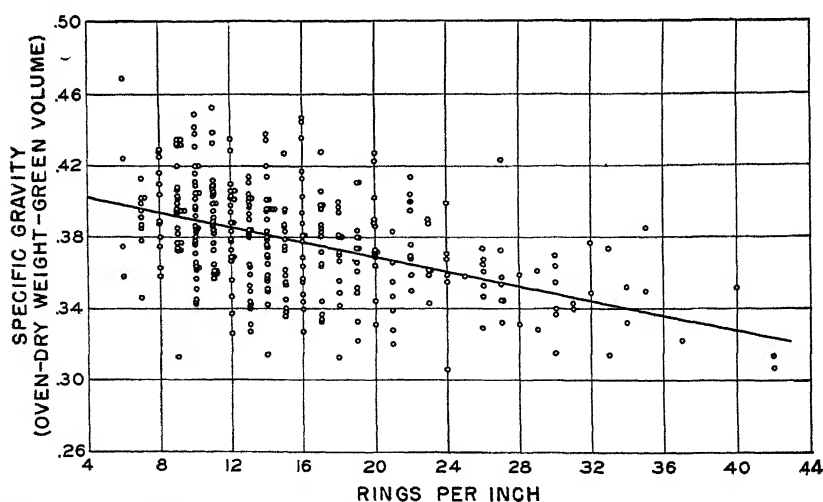
FIGURE 2.—Relation of specific gravity to rings per inch for the blocks from 23 yellow poplar trees.

errors of estimate. The chances are about 95 out of 100, therefore, that a specific gravity value, estimated from the first equation for a given value of rings per inch, will not be in error by more than ±0.054 (twice the standard error).

A statistical summary of the data is presented in table 1.

TABLE 1.—*Analysis of covariance of rings per inch and specific gravity, together with regression equations and correlation coefficients*

Source of variation	Degrees of free-dom	Sum of squares	Mean square	Regression equation	Correla-tion co-efficient	Degrees of free-dom
General regression	1	0 0742	0 0742	$Y=0.412-.0020X$	−0. 483**	343
Between tree regressions	22	.0150	.00068**			
Between tree means	22	.1242	.00565**	$Y=.408-.0018X$	−. 290	22
Error (within trees from individual regressions)	299	.1043	.00035			
Between tree regressions+error (within trees from average regression)	321	.1194	.00037	$Y=.413-.0021X$	−. 588**	321

**Highly significant.

The range of the correlation coefficients for the blocks of the individual trees was 0.479 to −0.893. The range for the regression coefficients was 0.0024 to −0.0070. Twenty of the coefficients were negative values; 3 were positive. These values show the great variability found between trees in the relation of rate of growth to specific gravity.

The average specific gravity, including heartwood and sapwood, was 0.378. The standard deviation was ±0.030.

The correlation between means of trees was low (−0.290). Testing by the null hypothesis shows this value to be below the 5-percent level, or nonsignificant, which indicates that there is no reliable association between the average specific gravity of each of the trees and their average values of rings per inch. This conclusion is confirmed by an analysis of error variance of the sum of squares of tree means of specific gravity, Sy^2, and reduction of Sy^2 due to regression. The F value of mean square of reduction divided by the mean square of the difference is nonsignificant. Hence, the different average values of growth rate of the trees did not account for any significant amount of the variance in average specific gravities.

To determine whether the average growth rates of the trees were significantly different, rings per inch, X, were tested by analysis of variance. The F value obtained proved that the original tree means rings per inch were highly significantly different from each other. It was found further that when analyses of variance was applied to the actual tree means of specific gravity, Y, those means were highly significantly different. After the means of specific gravity were adjusted to an average value of rings per inch, they were still highly significantly different, as is shown in table 1.

The mean square of the variation between tree means of specific gravity is the average variation between the means after they are adjusted to an average level of growth rate, X. When compared to experimental error with 321 degrees of freedom by the F test, this mean square (0.00565) is found to be highly significant. This indi-

cates that the tree means of specific gravity do not change regularly
with mean rings per inch, but are definitely erratic. The growth rate
does not explain the differences among tree mean specific gravities;
after the mean specific gravities of the trees are adjusted to an aver-
age growth-rate basis, they still differ significantly from each other.
Evidently, other biological forces influence the average specific grav-
ity of a tree more than does the average growth rate within the range
studied. One reason for this may be a lag in response of specific
gravity to changes in growth rate, although a study of the data of
the individual tree samples does not place much weight on this factor.
Age may have a small effect in some trees, as will later be shown. It
seems likely that inherent or hereditary characteristics may be respon-
sible to a large degree for wide differences in adjusted mean specific
gravities of trees, granting that ecological factors would exert a modi-
fying influence.

The differences between tree means also are responsible for some
of the total variance between rings per inch and specific gravity when
all the blocks are considered as a random sample as in figure 2. The
low between-tree correlation coefficient, with its attendant large
sum of squares of error of estimate resulting from variation of tree
means, offsets the higher within-tree coefficient to give the interme-
diate coefficient of total correlation.

The average correlation coefficient (the appropriate average of the
23-tree correlation coefficients), based on the average within-tree
regression, is −0.588. Correlation of rings per inch to specific gravity
within the trees after eliminating the effect of differences among the
tree means of rings per inch and specific gravity, is considerably
better than that for the mean values of the trees. However, since
rings per inch, in average regression, accounts for only 34.5 percent
(r^2) of the variance in specific gravity within trees, this relationship
is fairly low.

The above considerations indicate that for flat-sawn inch boards
selected at random, some relation between rings per inch and specific
gravity for the individual pieces would be expected ($r=0.483$). For
thicker cuts, less association would be probable, and for large timbers,
very little or no significant correlation would be expected between
the mean values of the individual timbers, since the larger cuts more
nearly approximate the conditions of the separate trees in which a
lower correlation of −0.29 exists.

The mean square of between-tree regressions (line 2, table 1) is
an average of the variation in specific gravity that is not explained
by the regular effect of changing growth rate within each tree upon
the specific gravity. The individual tree coefficients of regression
or rates of change of specific gravity with rings per inch are signifi-
cantly different from each other and from the average regression or
slope. An average slope, such as that indicated by the regression
coefficient in the third equation in table 1, does not give a true repre-
sentation of all the individual trees. The individual slopes shown
in figure 3, differ from the best average slope for the group more
than would be expected from trees taken from a homogeneous popu-
lation. There appear to be inherent differences between the trees
that cause some of them to be rather unlike others in this relationship

of growth rate to specific gravity. This difference, however, is apart from the differences in levels of the curves, i. e., the differences in the tree means of Y (specific gravity).

The sum of squares of errors of estimate, 0.015 with 22 degrees of freedom, is associated with the variation of the tree regression coefficients, or the failure of the individual tree regressions to coincide with the average within-tree regression. This is the difference between the sum of squares of errors of estimate for variation within trees from average regression and the corresponding value for variation within trees from individual regression. The mean square 0.00068 for regressions is tested by the F test against the mean square of deviations from individual tree regressions with 299 degrees of freedom (line 4, table 1). The mean square of deviations is an average of the variation in specific gravity not explained by tree differences of X and Y or by individual regressions. The quotient was found to be highly significant. Hence, an average slope of curve does not adequately represent the individual slopes.

The regression line for each tree is shown in figure 3. This figure illustrates the variability discussed above regarding the differences between the slopes of curves for the individual trees, the differences between levels of the curves, the range of rings per inch and specific gravity for each tree, and gives the correlation for the regression line of each tree.

The first equation in table 1 (column 5) represents the curve shown in figure 2 for the total correlation where Y is specific gravity and X is rings per inch. From a practical standpoint, this is probably the equation of most importance because it represents the average relation between rings per inch and specific gravity that might be expected if samples were taken from a pile of lumber. The second equation expresses the curve that would be obtained for weighted mean values of rings per inch and specific gravity for the individual trees. The

CORRELATION COEFFICIENTS

A −.44	J −.68	S +.10
B −.65	K −.50	T −.45
C −.60	L −.72	4 +.48
D −.58	M −.50	5 −.58
E −.42	N −.17	6 −.73
F −.84	O −.89	7 −.16
G −.83	P −.78	9 −.79
H −.75	R +.03	
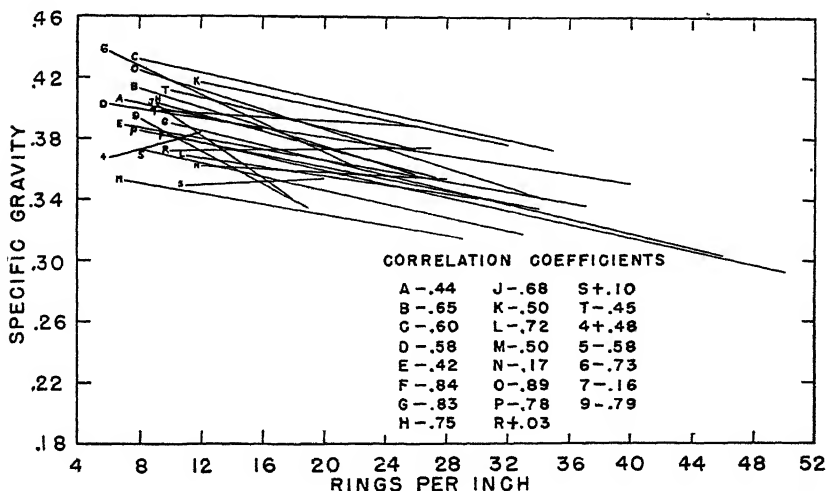
FIGURE 3.—Individual regression lines and correlation coefficients for specific gravity and rings per inch of 23 yellow poplar trees.

third equation expresses the average of the individual tree regressions shown in figure 3. This equation is based on the means of X and Y for all the samples and the average within-tree regression coefficient for the 23 trees. It represents the average relations between rings per inch and specific gravity that exists within the trees after correcting for the differences among the means of the trees.

In the light of the results recorded above, it is evident that the biological forces which cause a within-tree relationship are not those which cause different trees to have different specific gravities.

II. RINGS PER INCH AND VOLUMETRIC SHRINKAGE

Figure 4 shows the experimental data for rings per inch and volumetric shrinkage of all the blocks. The standard error of estimate of volumetric shrinkage for the total regression is ± 0.961 percent. An inverse relationship was obtained, as would be expected, because of the inverse association of rings per inch with specific gravity (fig. 2).

A statistical summary of the data is given in table 2.

The mean square for differences between tree regressions tested against experimental error (item 4) shows that these regressions or slopes are significantly different from each other, although the probability value is just at the 5-percent level. This shows less variation among the slopes than was found for rings per inch and specific gravity. The tree slopes are so similar that they can almost be considered as coming from a uniform group and as fitting quite well an average slope.
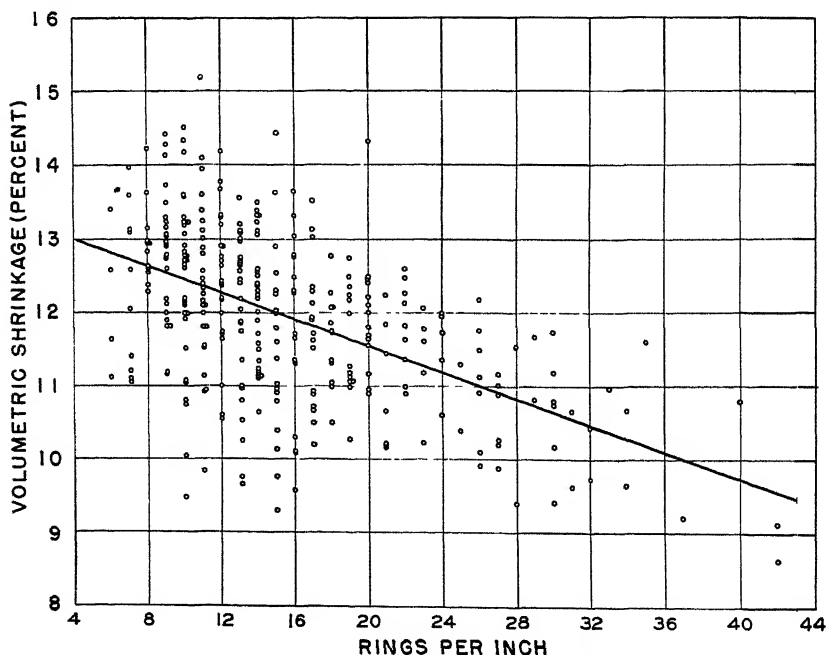
FIGURE 4.—Relation of volumetric shrinkage to rings per inch for the blocks from 23 yellow poplar trees.

TABLE 2.—*Analysis of covariance of rings per inch and volumetric shrinkage together with regression equations and correlation coefficients*

Source of variation	Degrees of freedom	Sum of squares	Mean square	Regression equation	Correlation coefficient	Degrees of freedom
General regression	1	143 97	143 97	$Y = 13.37 - 0913 X$	−0. 560**	339
Between tree regressions	22	23. 18	1.053*			
Between tree means	22	125. 32	5. 696**	$Y = 14\ 39 - .1557\ X$	−. 665**	22
Error (within trees from individual regressions).	295	166. 51	.564			
Between tree regressions+error (within trees, from average regression).	317	189.49	.598	$Y = 13\ 13 - .0749\ X$	−.538**	317

*Significant.
**Highly significant.

The tree-mean volumetric shrinkage varies with mean growth rate in a different way than does volumetric shrinkage of blocks within a tree with growth rate. As shown in table 2, the mean square for tree means of volumetric shrinkage is highly significant as compared with error (line 5). These adjusted tree means differ more than would be expected from chance; tree mean values of volumetric shrinkage deviate significantly from the average regression. In other words, the differences in tree-mean values of volumetric shrinkage are not entirely accounted for by the average regression curve.

Tree-mean shrinkage does change to some extent with mean growth rate because the variance of Y was reduced about 44 percent by the regression of Y on X. In this case, the trend is better than that of the other regressions. The equation of the means shows a regression coefficient of −0.1557 as against −0.0913 for general regression and 0.0749 for average within-tree regression. The correlation coefficient for tree means was −0.665 as compared to −0.560 for total correlation and −0.538 for average correlation. This is the opposite of the results obtained in the rings-per-inch vs. specific-gravity analysis (section I), in which the tree-mean correlation was the lowest of the three coefficients. The tree means fit their regression line better than all the blocks fit their average regression within trees.

The coefficients of regression for the individual trees ranged from +0.0310 to −0.2570 and the correlation from +0.184 to −0.882. All the trees gave negative coefficients except one.

Further details on procedure of analysis and interpretation can be made in a manner similar to that given in the preceding section on rings per inch and specific gravity.

III. RINGS PER INCH AND TANGENTIAL SHRINKAGE

An examination of figure 5 reveals a better association between the variables than was shown for the relationships discussed in sections I and II. The higher correlation may be accounted for in part by the method of measurement of the tangential dimension. The dial-gauge caliper measures only a small width and area. In measuring the green block in the tangential direction one central spot was marked to assure remeasurement in the same place after drying. The ring width was not uniform in some blocks. Hence some measurements of
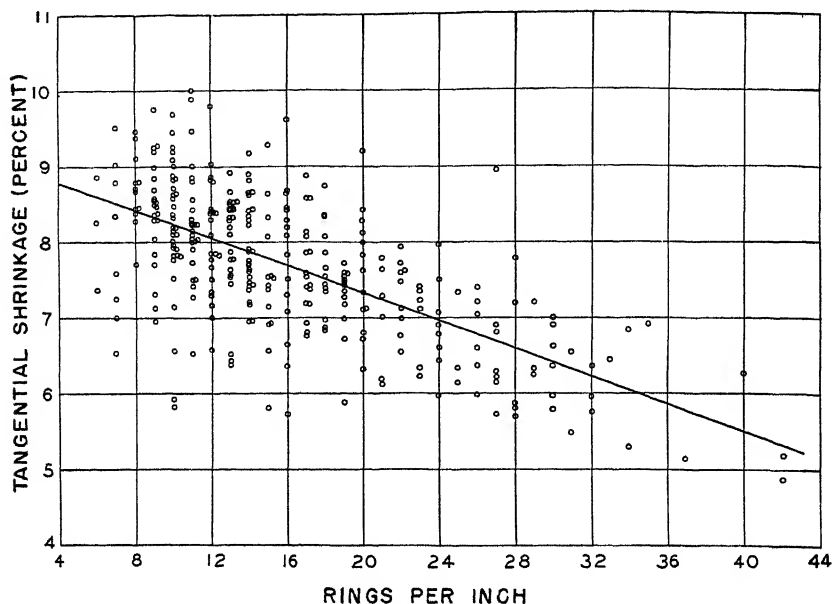
FIGURE 5.—Relation of tangential shrinkage to rings per inch for the blocks from 23 yellow poplar trees.

shrinkage and rings per inch represented values for a narrow-ring zone or for a wide-ring zone. If the measurement is in the center of the zone the effect of ring width should be at a maximum; there should be less counteraction of shrinkage by the adjacent zone of different ring width. It was observed that on the surface of the dried blocks the wide-ring zones were depressed below the level of the narrow-ring zones when these zones alternated in the same block. The difference in level was not always abrupt regardless of the change in ring width. The standard error of estimate of tangential shrinkage for the total regression is ±0.723 percent.

The statistical data are given in table 3.

TABLE 3.—*Analysis of covariance of rings per inch and tangential shrinkage, together with regression equations and correlation coefficients*

Source of variation	Degrees of freedom	Sum of squares	Mean square	Regression equation	Correlation coefficient	Degrees of freedom
General regression	1	148.44	148 44	$Y=9.15-0.09X$	-0.676**	337
Between tree regressions	22	11.19	.509			
Between tree means	22	67.53	3 05**	$Y=10.07-.148X$	-.713**	22
Error (within trees from individual regressions)	293	97.84	.334			
Between tree regressions + error (within trees, from average regression)	315	109.03	.346	$Y=8.89-.075X$	-.652**	315

** Highly significant

The range of individual tree regression coefficients was +0.001 to −0.198. The range of correlation coefficients was ±0.009 to 0.921; the average correlation as given in table 3 was −0.652. The tree

regressions or slopes were not significantly different from each other and are, therefore, quite accurately represented by the average regression (third equation, column 5). The correlation between tree means is higher than either the within-tree or the total correlation. This finding is similar to those obtained for rings per inch and volumetric shrinkage.

The data for blocks of 20 rings per inch or more were analyzed separately. The correlation coefficient for this slower growth was −0.66. This indicates about the same correlation for wood of slower growth as for all growth rates (−0.676) on a total correlation basis.

IV. RINGS PER INCH AND RADIAL SHRINKAGE

The correlation of the two variables rings per inch and radial shrinkage was determined on the basis of tree mean values by giving equal weight to each tree, and not on the individual block data. The coefficient obtained as −0.166, a value so small that for the number of trees used it has no significance.

V. SPECIFIC GRAVITY AND VOLUMETRIC SHRINKAGE

The relation between specific gravity and volumetric shrinkage for the blocks is shown in figure 6. The standard error of estimate of volumetric shrinkage is ±0.823 percent. The total correlation coefficient for the statistics shown in figure 6 is −0.734. It follows that about 54 percent of the variance in volumetric shrinkage of the blocks from their mean can be explained by differences in specific gravity. This is considerably better than was found for rings per inch and volumetric shrinkage. Twenty-four trees were used in this part of the study.

A summary of the data obtained is given in table 4.

TABLE 4.—*Analysis of covariance of specific gravity and volumetric shrinkage, together with regression equations and correlation coefficients*

Source of variation	Degrees of freedom	Sum of squares	Mean square	Regression equation	Correlation co-efficient	Degrees of freedom
General regression	1	281.21	281.21	$Y = 0.941 + 29.08X$	0.734**	355
Between tree regressions	23	18.76	.82**			
Between tree means	23	97.87	4.26**	$Y = .941 + 29.08X$	.734**	23
Error (within trees from individual regressions)	309	123.70	.40			
Between tree regressions+error (within trees, from average regression)	332	142.46	.43	$Y = .933 + 29.09X$	.735**	332

**Highly significant.

The range of individual tree coefficients was 9.00 to 51.66 for regression and 0.372 to 0.961 for correlation. The averages of within-tree values as given in table 4 were 29.09 and 0.735 respectively. These averages, however, are only indicative of the trend of this particular experiment since, as is explained below, they are based on a population that is not homogeneous.

The mean square for the error of estimate of between-tree slopes or regressions is highly significant as compared with error by the F test, indicating that the relation of specific gravity to volumetric shrink-
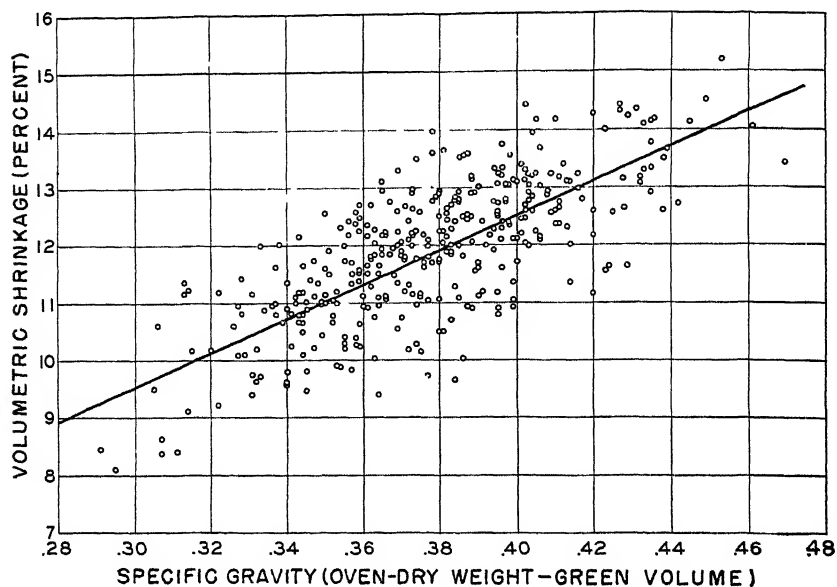
FIGURE 6.—Relation between volumetric shrinkage and specific gravity for the blocks from 24 yellow poplar trees.
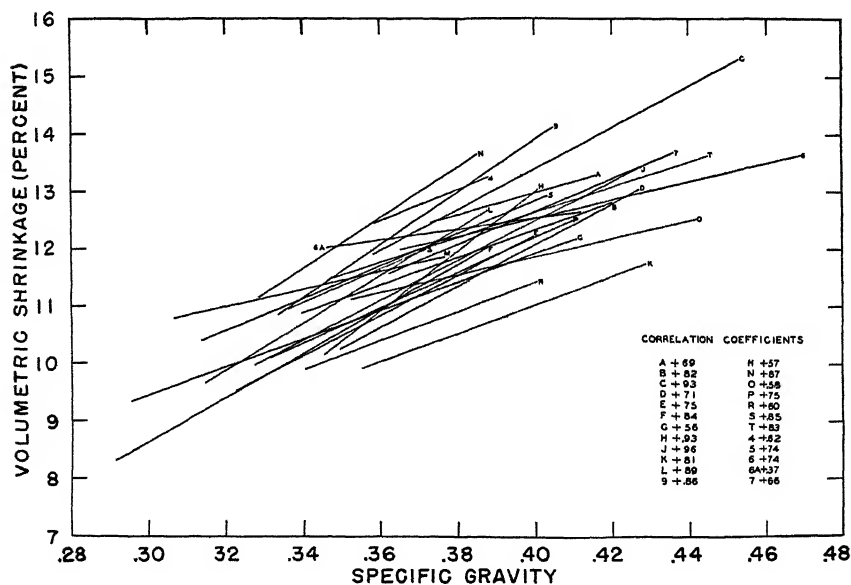
FIGURE 7.—Individual lines and correlation coefficients for volumetric shrinkage and specific gravity of 24 yellow poplar trees.

age is different for the various trees, and the differences in slopes are more than would be expected on the basis of chance from a sample of trees which had curves of common slope. A very significant difference is shown also between the tree regressions, based on their departure from the average within-tree regression. No common slope can fully represent the individual slopes to the extent of accounting for the regression differences from it that would not be due to chance.

The mean square of 4.26 for tree means indicates a highly significant difference between tree means in volumetric shrinkage after adjusting them to an average level of specific gravity. The tree means, therefore, differ more among themselves than would normally be expected from a homogeneous group.

It is interesting to note that the three correlation coefficients in table 4 are practically the same, a feature not found in other analyses.

Figure 7 shows the individual regression lines for specific gravity and volumetric shrinkage. The graph shows the different slopes, the various levels of the slopes, the ranges of the two variables for each tree, and the correlation coefficients for each tree.

In order to check the validity of the use of a straight line for the composite data (fig. 5) as well as to determine the nature of the curves of the individual trees, the data on specific gravity and volumetric shrinkage were plotted for each tree. It was evident from the individual graphs that a straight line properly represented the relation between the two variables in nearly every case and would best express the average relation between all trees.

VI. SPECIFIC GRAVITY AND TANGENTIAL SHRINKAGE

Figure 8 presents graphically the data for relation between specific gravity and tangential shrinkage for all blocks (except near the pith). The regression line shown represents a total correlation coefficient of 0.676. This indicates less association than was found for volumetric shrinkage. The standard error of estimate of Y (tangential shrinkage) for these data is ± 0.727 percent.

A summary of the data is given in table 5.

TABLE 5.—*Analysis of covariance of specific gravity and tangential shrinkage, together with regression equations and correlation coefficients*

Source of variation	Degrees of freedom	Sum of squares	Mean square	Regression equation	Correlation coefficient	Degrees of freedom
General regression	1	148.86	148.86	$Y=0.709+22.18X$	0.676**	334
Between tree regressions	22	9.25	.42*			
Between tree means	22	91.09	4.14**	$Y=.384+19.28X$	.590**	22
Error (within trees from individual regressions)	290	76.42	.26			
Between tree regressions+error (within trees, from average regression)	312	85.67	.27	$Y=1.51+24.31X$	.739**	312

* Significant.
**Highly significant.

The range of individual tree regression coefficients was 10.83 to 39.35; for correlation coefficients the range was 0.218 to 0.937. Applying the F test, it is found that the mean square 0.42 is just at the
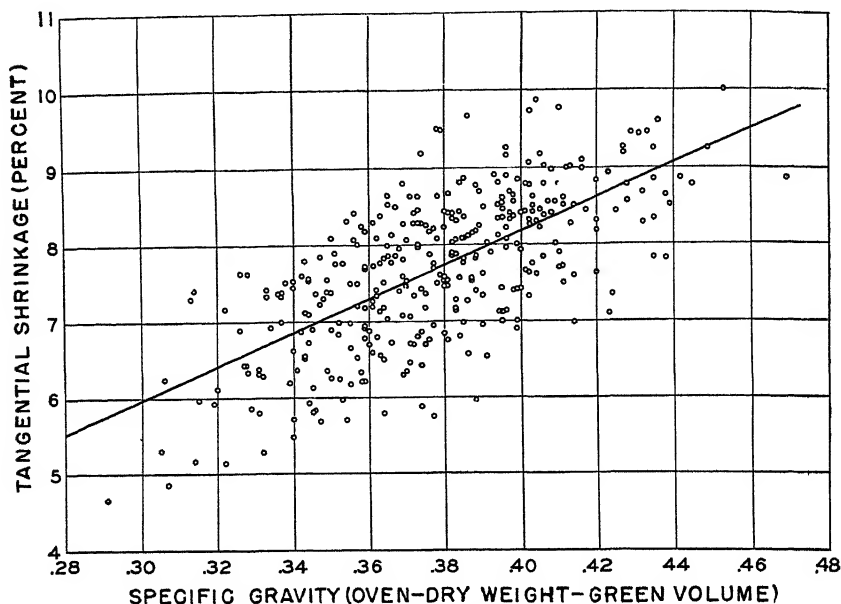
FIGURE 8.—Relation of tangential shrinkage to specific gravity for the blocks of 23 yellow poplar trees.

5-percent level of significance; therefore, the tree regressions differ but little from each other and from their average regression.

The adjusted tree means of tangential shrinkage are highly significantly different from each other. The total variation of tangential shrinkage not accounted for by specific gravity is in part caused by tree-to-tree differences, as is indicated by the fact that the correlation coefficient for tree means (0.590) is lower than the average within-tree correlation (0.739).

VII. SPECIFIC GRAVITY AND RADIAL SHRINKAGE

The association between specific gravity and radial shrinkage was not nearly so good as for the other shrinkages. The summary of data is given in table 6.

TABLE 6.—*Analysis of covariance of specific gravity and radial shrinkage, together with regression equations and correlation coefficients*

Source of variation	Degrees of freedom	Sum of squares	Mean square	Regression equation	Correlation coefficient	Degrees of freedom
General regression	1	25.65	25.645	$Y=0.715+9.261X$	0.487**	330
Between tree regressions	22	6.298	.286**			
Between tree means	22	40.311	1.832**	$Y=.838+8.935X$	.448*	22
Error (within trees from individual regressions)	286	36.083	.126			
Between tree regressions+error (within trees, from average regression)	308	42.379	.137	$Y=.624+9.502X$	.518**	308

*Significant.
**Highly significant.

The standard error of estimate of radial shrinkage in the general regression is +0.500 percent.

The range of coefficients for individual trees was −0.251 to +0.929 for correlation and −3.326 to +22.371 for regression. It is obvious that the association between specific gravity and radial shrinkage is low. The best group relationship was found with the average within-tree regression, where 27 percent of the variance of radial shrinkage was accounted for by variation in specific gravity (r=0.518). Since the tree regressions are not homogeneous, this value is merely indicative of the average for these data.

Differences between the adjusted mean values of radial shrinkage for the individual trees are highly significant. The correlation coefficient for the tree mean values of specific gravity and radial shrinkage (0.448) is between the 5-percent and 1-percent levels of significance. This level of significance coupled with the relatively low correlation gives this relationship only minor practical importance.

VIII. AGE AND SPECIFIC GRAVITY ·

The association of specific gravity with age as indicated by the number of rings from the pith was determined by grouping the blocks into rings-per-inch classes of 1 to 10, 11 to 20, 21 to 30, 31 to 40, and over 40. The blocks in the first class actually had from 6 to 10 rings per inch. The apparent effect of age was greatest in the 1 to 10 class and decreased to an absence of effect in the slow-growth samples. The curves are shown in figure 9.

The correlation index for the 1–10 class (containing 101 blocks) was 0.62, which accounts for 39 percent of the variance. Included in this class were 27 blocks of 20 years of age or less and when these were excluded from the analysis, the correlation index was 0.45. Considerable scatter existed over the whole range of the curve. Three-fourths of the blocks above 20 years of age were from 9 trees only; only 2 blocks were over 200 years old.

In the 11 to 20 curve there were 211 points representing all trees fairly well, but the deviations were marked. Twenty of the blocks had average ages of 5 to 20 years. The correlation index for this curve was 0.37.
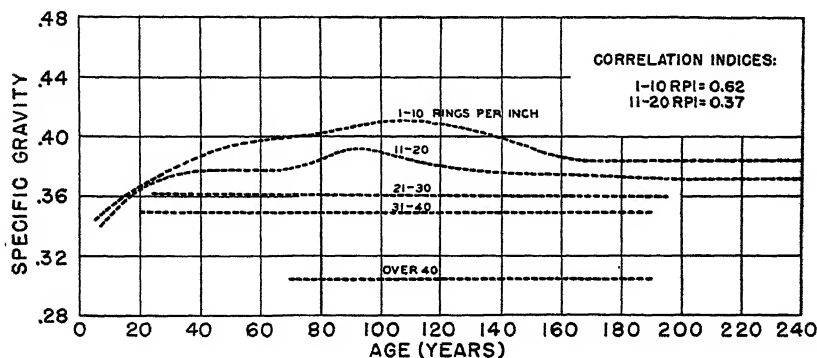
FIGURE 9.—Relation of specific gravity to age of wood. The blocks from 23 yellow poplar trees were grouped into growth-rate classes.

The curve for the 21 to 30 class was based on 53 blocks from 18 trees, the 31 to 40 curve on 12 blocks from 7 trees, and the class over 40 years on 7 blocks from 3 trees.

The wide dispersion of the points about the curves, together with the inadequate number of points and trees represented in some curves, as may be seen from the data given, makes the reliability of these curves questionable. This observation applies especially to the low-age area of the 1 to 10 and the 11 to 20 curves, which show a steep slope and marked deviations, and also to the two bottom curves, for which too few data were available. All the curves as drawn are the best fit of the experimental data. The limits of each curve were determined by the extremes of the points involved. It would be desirable to have this relationship of age and specific gravity checked by additional investigations.

The effect of age was also computed by a different method. The specific gravity for the wood in the outer half of the tree was determined for two classes of trees, one consisting of 17 trees less than 180 years of age at test height, the other of 6 trees more than 180 years of age and averaging about 225 years. The average rings per inch and specific gravity in the younger age class were 19.8 and 0.386, respectively, and for the older class, 17.8 and 0.362. The difference between these specific gravity means was found to be significant by the analysis-of-variance test.

IX. WOOD NEAR THE PITH

As previously stated, blocks averaging less than 2 inches from the pith or those less than 20 years of age were not included in the analysis of data and relationship of properties. The shrinkage and specific gravity values for this wood were computed separately from the wood that lay beyond. The data for each type are given in table 7.

TABLE 7.—*Specific gravity and shrinkage of wood near the pith and of wood away from the pith*

Position and age of wood	Average specific gravity	Average shrinkage			
		Volumetric	Tangential	Radial	Longitudinal
Less than 2 inches from pith and less than 20 years old	0.348	*Percent* 11.71	*Percent* 7.61	*Percent* 3.77	*Percent* 0.17
More than 2 inches from pith and more than 20 years old	.378	11.92	7.67	4.20	.20

The important differences shown in table 7 are in specific gravity and radial and longitudinal shrinkages. Volumetric and tangential shrinkages are about the same for the two groups in spite of the fact that specific gravity was lower for the first group. In other words, these shrinkages of the wood near the pith are greater than would be expected from their normal relation to specific gravity. This was observed also when these data were plotted on the same graph with the data of the wood away from the pith. The points for the wood near the pith were usually in the upper portion or above the other points for a given specific gravity.

To determine the approximate distance from the pith at which the density of the wood became normal, the average specific gravity of the blocks just outside the limits given above was computed. The value obtained was 0.370 which, when compared with 0.378 (table 7), indicates that this wood was about normal. The upper two curves in figure 9 illustrate this point. Examination of the data for the individual trees did not indicate that the lower specific gravity was due to unusually wide rings although these were present in a few cases. The lower specific gravity was found even in wood of the same or a slower rate of growth than that of subsequent years in the same tree. Nor was it true that all trees had the same amount of low-density wood near the pith. In a few cases the wood less than 20 years old and less than 2 inches from the pith was about as dense as the wood subsequently formed. In other cases the wood just beyond this zone was somewhat below normal in density as compared with later wood having approximately the same number of rings per inch. Since the age and distance limits used in this test are the average values for the test piece (usually at the midpoint), no exact limit of age or distance was actually used. However, considering the averages already given and the rings of each tree, it was concluded that for these trees a radius of 2 inches from the pith included practically all the wood near the pith that was subnormal in specific gravity. Variation about this arbitrary limit is to be expected and does exist. Wood with a rate of growth near the pith of 8 to 16 rings per inch (which was the rate for three-fourths of the trees) usually will be 20 years of age or slightly over. These variations are not shown in detail in the curves of figure 9 since it was feasible to show only the average relationship.

X. EFFECT OF CHANGING GROWTH RATE

The range in rate of radial growth was 0.37 inch to 1.5 inches for the first 10 years. The influence of the initial rate of growth at the test height on the specific gravity of the wood subsequently formed was determined by partial correlation of the factors: rings per inch in the first 10 years, average rings per inch, and average specific gravity. The last two factors applied to the wood along the diameter and over 20 rings from the pith.

With the effect of different average rates of growth of the tree eliminated, it was found that the partial correlation coefficient for the rate of growth in the first 10 years and the average specific gravity was −0.157. It was concluded that the growth rate in the first 10 years had no significant influence on the average specific gravity (based on oven-dry weight and volume when green) of the wood subsequently formed or beyond approximately the 20-year-old zone.

Seventeen trees had a period of slow growth (20 or more rings per inch) at some time during their lives, usually about the middle period. In general, the specific gravity dropped to a varying degree in the slow-growth period, and upon the resumption of more rapid or normal growth, it returned to normal or to a value similar to that before the slow growth. This seemed to be true whether the tree had a low, intermediate, or high average specific gravity and irrespective of the rate of growth for the first 10 years, within the range of rate of growth that was found in the experiment.

XI. CROWN-SURFACE RELATIONSHIPS

The area of the crown surface was calculated by the equation for the area of a semiellipse whose semiaxes are a and b, where a and b represent crown depth and average crown radius, respectively. This may not give the exact effective area for a number of reasons, such as irregularity of crown shape, variation in leaf density of the crown, the unknown relation of crown size to photosynthetic activity, etc.; but for want of a better method the above formula was used. The average annual cross-sectional increment in square inches for the last 10 years was computed for each tree, based on the cross section at the end of the first log. These values were correlated with the crown-surface area. The correlation coefficient was 0.60. This exceeds the 1-percent level and therefore is regarded as highly significant. The coefficient of determination indicates that 36 percent of the increment variance was due to differences in crown area.

When the average cross-sectional increment for the last 30 years was used, instead of the increment for the last 10 years, the correlation coefficient was 0.55 as against 0.60 for the 10-year period. Average crown diameter and the cross-sectional increment for the last 10 years gave a correlation coefficient of 0.57. Crown length and area increment gave a coefficient of 0.54. Multiple correlation for the three factors—cross-sectional increment, crown diameter, and crown length—gave a coefficient of 0.59, which is almost the same as that obtained for increment and the crown area, based on the formula of a semiellipse. This is another way of showing that crown diameter and crown length augment each other to only a small degree in accounting for differences in growth increment. If the product of crown diameter and crown length for each tree is plotted against the cross-sectional increment, a correlation coefficient of 0.59 is obtained, which is the same as that obtained in multiple correlation and approximates the coefficient of 0.60 for crown-surface area and cross-sectional increment. The small differences between the coefficients derived from crown area, multiple correlation, the product of crown diameter and crown length, and crown diameter indicate that, for most purposes, crown diameter is to be preferred to crown area as a measure of area of growth increment because fewer measurements and computations are involved.

No adjustment was made in the foregoing data for variation in bole length. An attempt was made to correct for this by computing the approximate bole surface (inside bark, including the height to base of crown) of each tree and the annual radial increment for the last 10 years. This volume increment correlated with crown-surface area gave a coefficient of 0.22. When volume increment was correlated with crown diameter the coefficient was 0.42. Why these correlations for volume increment should be lower than those for cross-sectional increment is not apparent. Possibly the trees with larger crowns put much of the growth increment on the branches and upper bole as well as on the bole below the live branches, or perhaps the volume increment figures are too inaccurate to give entirely dependable results, since the growth rate at various heights and the taper of the tree were not measured on each tree.

There was no significant correlation when rings per inch for the last 10 years were plotted against crown-surface area. There did not appear to be a definite relation between the crown size and the specific gravity of the wood in the outer half of the tree growth, even when the variation in sizes of the tree trunks was taken into account.

XII. PARTIAL CORRELATION ANALYSIS

The coefficients of partial correlation were computed for the variables rings per inch, specific gravity, and volumetric shrinkage. Volumetric shrinkage was used as the dependent variable, or the property upon which the influence of one factor was to be measured while the effect of a third factor or property was eliminated.

The correlation between volumetric shrinkage and specific gravity when rings per inch was held constant was 0.641. The drop from 0.734 for gross correlation to 0.641 for partial correlation indicates that as the rate of growth varies, it has some effect on the true correlation of specific gravity with volumetric shrinkage.

The partial coefficient of correlation between volumetric shrinkage and rings per inch with the influence of specific gravity eliminated was −0.338. The gross coefficient of determination was 0.314 and the partial coefficient 0.114. This relatively large decrease means that the gross correlation of volumetric shrinkage with rate of growth was largely a relationship between specific gravity and volumetric shrinkage, expressing itself, in part, as a rate-of-growth influence.

When the aggregate relationship of these three variables was calculated, it was found that the coefficient of multiple correlation was 0.77, or slightly higher than the original gross correlation between specific gravity and volumetric shrinkage. That the value is not higher is due to the small effect of rings per inch on the shrinkage, as shown above. The multiple correlation indicates that the factors of rings per inch and specific gravity together account for about 55 percent of the variance of the volumetric shrinkage.

XIII. SPECIFIC GRAVITY OF WOOD FROM NORTH AND SOUTH SIDES OF TREE

The average specific gravity of the wood from the north and south sides of each tree was obtained from the data on the blocks on each side, sapwood and heartwood included (except the zone 2 inches from the pith). Equal weight was given for each tree in computing the averages for all trees, since the desired result is concerned with the situation among trees even though they are not of the same size. The average number of rings per inch for each side was approximately the same (17 and 18). The average specific gravity for the north sides was 0.375 and for the south sides 0.377. The two values are not significantly different. In 11 trees the wood on the north side had the higher specific gravity, and in 12 trees, the wood on the south side had the higher specific gravity. The range of ratios of the higher specific gravity to the lower was 1.01 to 1.12 when wood from the north side had a higher specific gravity than wood from the south side, and 1.01 to 1.09 when the reverse was true. The mean in both cases was 1.04.

The average specific gravity of a particular side seemed to be associated with the length of the radius (north or south) of the trees.

In 11 trees the side which had the higher specific gravity was the side of shorter radius, and in 5 trees the side of higher specific gravity was the side of longer radius. In the remaining 7 trees, there was either no appreciable difference in specific gravity between the two sides (less than 2 percent) or else the two radii were nearly the same. Of 8 trees in which one radius was 20 percent greater than the other, 5 had the longer radius on the north side and 3 on the south side. Four of the 8 were heavier on the shorter side, 2 were lighter on the shorter side, and 2 showed no appreciable difference in specific gravity.

●   XIV. LONGITUDINAL SHRINKAGE

The average longitudinal shrinkage for all the blocks from all the trees (except wood near the pith) was 0.20 percent. The average shrinkage for the wood on the north side of the trees was 0.21 percent and for that on the south side 0.18 percent. The maximum average shrinkage found along a radius in any tree was 0.36 percent. In 14 trees the average longitudinal shrinkage was greater on the side of the lower specific gravity. In only 1 tree was there higher longitudinal shrinkage on the side of higher specific gravity. In the remaining 8 trees the shrinkage was about the same on both sides, or else the specific gravities were nearly the same. The relative length of the north and south radii on each tree also seemed to be associated more or less with longitudinal shrinkage. In 15 trees, the side of longer radius had the greater longitudinal shrinkage, but the ratios for 5 of these were small. In 7 trees the side of shorter radius had the greater longitudinal shrinkage, but in only 3 of these was the relation definite. These results agree with those recorded in section XIII in which it was shown that in 11 trees the side of shorter radius had the higher specific gravity, in 5 the reverse was true, and in 7 either the specific gravity ratio or the radius ratio was so small that the data were neutral. High or low longitudinal shrinkage did not seem to be associated with fast or slow growth or with deviation from the normal in other respects. Since the shrinkage values are not unusual, associated factors may not be present.

The above results are not considered conclusive but are presented as an observed trend which should be checked by other work.

When longitudinal shrinkage was plotted against specific gravity, using the average values of north and south sides, no significant correlation resulted. This comparison presents a different picture from that obtained by comparing ratios of the two sides of a tree. Trees may have a similar north-to-south ratio but be very different in specific gravity in whole or in part.

DISCUSSION

One of the principal reasons for making the statistical analyses presented herein was to determine the reliability and the closeness of association between the various factors that affect specific gravity and shrinkage in wood. It has been common practice to say that certain factors influence specific gravity and shrinkage and either to ignore or to give inadequate evaluation to the usefulness and reliability of the relationship of these factors. For example, there is

general agreement in the literature that the rate of growth has an influence upon specific gravity; but there is very little information to show how much of the variation in specific gravity is due to or is associated with number of rings per inch. A procedure such as the one used in this experiment gives a measure of this association and also provides information on the significance of some factors that contribute to variation.

Only one tree used in this study had an average growth rate as fast as 8 annual rings per inch. This was a codominant which grew at the bottom of the slope near a creek. In the first 10 years this tree grew at the rate of 6 rings per inch; thereafter the rate was uniformly 8 rings per inch. The tree was 21.4 inches diameter breast high and 85 years old. The trees that made the next most rapid growth averaged, respectively, 10.5 and 13 rings per inch.

It is somewhat disturbing to find that so few of the relationships show a high degree of association of the variables involved. In these cases undetermined factors may be of major importance. This seems to be true with respect to rings per inch and specific gravity, for example. It is desirable, at this point, to emphasize the difference between one tree and another in the closeness of association of any two of the variables to be measured. It is evident from the wide range of the coefficients given for the different pairs of variables and from the significance of the mean squares that the relation of, say, specific gravity and volumetric shrinkage is very different from one tree to another and may be very different also from the average coefficient for the group of trees. The differences between these values show also that one cannot rely on a small number of trees for obtaining the measure of a variable factor upon a property of wood that will have sufficient reliability to give the results real worth. This is true also in measuring any single factor or property. In using an equation or a curve to derive one value when the other is known, the derived value represents the average that would be expected from a large number of samples having the same value as the X variable. However, if the best curve that can be made reduces the total variance of Y by only a small amount, say 25 percent or less, we must accept this fact and recognize the limitations of the equation (or curve) as a means of computing or predicting the values that we wish to use as actual values. In this connection the standard error of estimate of the dependent variable, Y, is of some use in indicating the probable limits of error of the estimated value.

Linear relationships have been used for most of the analyses reported in this study. Judging from the data, there seemed to be good reason for doing so; also, the use of linear relationships makes possible the analysis of covariance to evaluate the various factors reported. It is doubtful whether there are sufficient points in the range of less than 10 rings per inch to determine accurately the end of the curve that involves rate of growth. It may be that the curve should flatten out or even drop a little, as is indicated by certain workers (*2, 6*). In the present study it seems unlikely that the error of the curve, if any, would be more than the change of the ordinate value to the left of 8 rings per inch. The regressions have not been computed to pass through the zero points of the X and Y axes. Rather, the graphs

and statistical conclusions are based on the experimental data without holding to control points. The statistical values obtained should therefore be the best values for the data.

In order to hold to a minimum such variables as climate and soil, the sampling in this study was limited to one area. No positive statement is therefore justified in regard to the results that might have been obtained if trees from other areas had been included. Nevertheless, on the basis of the generally accepted evidence that there may be as much variation in the specific gravity of trees within one large area as between those from widely separated areas, the results of this study should give a fairly good idea of what might have been found had other areas been represented.

As previously mentioned, the test blocks were not taken from the same height in all trees, but the maximum difference of 10 feet in height probably had little influence on the results since butt wood was not used.

Since this project was started other work has been reported which has some relevance to this paper. Stern (7), in his report on the strength properties of yellow poplar, reports only a 2 percent greater average specific gravity of sapwood over heartwood and −5 percent for heartwood near the pith.

Luxford and Wood (2) found no significant difference in specific gravity between sound heartwood and sapwood. The above results are in good agreement with the facts revealed in this study, in which the specific gravity of the sapwood was 2.4 percent higher than that of the normal heartwood and the specific gravity of the wood near the pith was 7.7 percent lower than the normal heartwood.

In view of the fact that the difference between heartwood and sapwood was small and not highly significant, and taking into consideration the results of other workers, it is concluded that there is no important difference in specific gravity between the heartwood not near the pith and the sapwood of yellow poplar. The evidence indicates, however, that heartwood near the pith has a significantly lower specific gravity than heartwood away from the pith. The average specific gravity of 0.378 (based on volume when green and weight when oven-dry) was about the same as that reported by Stern (7) in the one tree used in his project, but was below the average reported by Paul and Norton (6) for second growth (0.392) and for young trees in old-growth stands (0.399). Actually, it was between these values and their value for old-growth trees (0.363). As they point out, their value of 0.392 includes the wood near the pith in trees originating in fairly open stands. If that were excluded the average specific gravity would be somewhat higher. The average specific gravity reported by Luxford and Wood (2), in a recent study involving 250 random specimens for specific gravity (86 of which were from West Virginia) was 0.38, on the basis of weight when oven-dry and volume when green; the average number of rings per inch was 18. The species average given by Markwardt and Wilson (3) is also 0.38; the average number of rings per inch was 14. The specific gravity of 0.378 reported in this paper was from blocks averaging 16 rings per inch.

The evidence presenting an average specific gravity of 0.38 for yellow poplar seems to be well substantiated. If the samples show a high

ratio of either very fast or very slow rate of growth a different average figure may be expected.

Table 8 presents a comparison of the shrinkage values of yellow poplar reported from several sources.

TABLE 8.—*Shrinkage values of yellow poplar*

Reference	Trees	Rings per inch	Specific gravity [1]	Shrinkage			
				Volumetric	Tangential	Radial	Longitudinal
	Number			*Percent*	*Percent*	*Percent*	*Percent*
Present report	23	16	0.378	11.9	7.7	4.2	0.20
Markwardt and Wilson (*3*)	11	14	.38	12.3	7.1	4.0	
Boomsliter (*1*)	1	14	[2].43		7.2	4.1	.15

[1] Based on green volume and oven-dry weight.
[2] Calculated from reported density of oven-dry material.

The differences among the various shrinkages are not large or important with the possible exception of tangential shrinkage. The method of measurement, as pointed out earlier, may account for the higher values obtained in this work.

SUMMARY

Some of the physical properties of yellow poplar (age 86 to 273 years) and some of the factors that might influence these properties were studied. Statistical analyses were used to evaluate these relationships and to indicate the interacting factors.

The specific gravity was found to vary inversely with the number of rings per inch but the correlation coefficient was small (−0.483). The difference between tree regressions and the difference between means of specific gravity of trees were found to be highly significant.

An inverse relationship was obtained between number of rings per inch and shrinkage—volumetric, tangential, or radial.

The variation between tree regressions for rings per inch and volumetric shrinkage and also between tree means of volumetric shrinkage were significantly different. The gross correlation was −0.560.

The total correlation for rings per inch and tangential shrinkage was 0.676. The tree means of tangential shrinkage were significantly different from each other.

The correlation of the tree mean values of rings per inch and radial shrinkage was small and nonsignificant (−0.166).

Volumetric shrinkage increased as specific gravity increased. The total correlation coefficient and the correlation coefficient between tree means were 0.734; thus, specific gravity accounted for 54 percent of the variance of volumetric shrinkage. The regressions for the individual trees and the mean values of volumetric shrinkage between trees varied more between themselves than would be expected from a uniform population.

The gross correlation for specific gravity and tangential shrinkage was 0.676. The tree regressions were significantly different from each other, and the tree means were also significantly different.

The gross correlation of specific gravity with radial shrinkage was 0.487, somewhat lower than with tangential shrinkage. Highly significant differences were found between individual tree regressions and also between tree means of radial shrinkage.

The total correlation was not usually the same as either the correlation of tree means or the average correlation within the trees.

Age was found to have some effect on specific gravity, especially in the blocks of 20 rings per inch or less. However, these results are not considered conclusive.

Wood near the pith was lower in specific gravity, radial shrinkage, and longitudinal shrinkage than wood farther out; in volumetric shrinkage there was little difference.

When the rate of growth changed in a tree there was usually an accompanying change in specific gravity, although the amount of change in specific gravity was quite variable. When the tree resumed its former rate of growth, specific gravity tended to return to its former value.

The initial rate of growth did not appear to influence the average specific gravity of the wood subsequently formed.

Crown-surface area correlated with the annual cross-sectional increment for the last 10 years gave a coefficient of 0.60. The correlation coefficient obtained where crown diameter was used instead of crown surface area was 0.572; with crown length the coefficient was 0.535. With calculated volume increment of the bole and crown-surface area, the correlation coefficient was 0.22.

No difference with respect to specific gravity was found between the north and south sides of the tree. There was some evidence that the side of shorter radius was more likely to have a higher average specific gravity than the opposite side.

The average shrinkage values, excluding wood near the pith, were: Volumetric 11.92 percent, tangential 7.67 percent, radial 4.20 percent, and longitudinal 0.20 percent. The average rate of growth was 16 rings per inch.

The average longitudinal shrinkage was generally greater in wood from the side of the lower specific gravity whether the wood was from the north or the south side of the tree. Likewise, wood from the side of longer radius usually showed a greater average longitudinal shrinkage than wood from the side of shorter radius.

The average specific gravity (based on oven-dry weight and volume when green) of 0.378, obtained in this study, is in good agreement with results obtained by other workers on yellow poplar.

Except in wood near the pith, no important difference was found between the specific gravity of sapwood and heartwood.

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DECAY RESISTANCE OF SEVEN NATIVE OAKS[1]

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INTRODUCTION

Oak is first among the United States hardwoods both in the volume available and in the volume used. This large acceptance is not entirely a result of the quantity at hand. Certain oak species have always been desired for their superior decay resistance and have been widely used for boat construction, ties, piling, bridge timbers and planking, rural-line poles, and other items commonly subjected to considerable decay hazard.

Oak species favored for decay resistance are generally specified commercially as "white oak" and are not otherwise distinguished. The other species are generally referred to simply as "red oak." This broad classification, which for the most part follows the botanical segregation of the species into white oak and red oak (or sometimes black oak) groups, has been convenient and apparently reasonably satisfactory. However, although it has been well established by general experience that the species of the red oak group usually do not last as long as those of the white oak group, there is little specific information to indicate how much inferior they may be in that respect. Moreover, there is practically no information about the relative decay resistance of the respective species within the two groups. Differences in decay resistance also occur within the same species of oak. One has but to observe the differences in service life of posts of a given species in the same fence line to be convinced of this. The more that is known about these differences in resistance both between and within species, the better will be the basis for selecting oak wood to meet varied durability requirements. Insofar as a choice can be made, it is often as inappropriate and as expensive to use wood of high durability on a dry site as it is to place nondurable wood on a

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damp site. In addition to selecting the most appropriate wood at hand to meet different service needs, the time may come when it will also be practicable to grow oak wood of superior decay resistance.

The purpose of the study reported herein was to ascertain by means of laboratory tests what differences in decay resistance exist (1) among some species of the commercially important oaks, (2) among trees of the same species in the same and different localities and regions, and (3) in different parts of the same tree. Samples were obtained from 407 trees. Of 375 trees that constituted the main basis of the study, 222 represented 4 species in the white oak group and 153 represented 3 species in the red oak group. The trees were distributed in 9 different regions in which most of the commercial oak is grown and in 2 or more rather widely separated localities in each region.

In appraising the information presented it should be kept in mind that laboratory decay tests introduce a factor of artificiality that precludes application of the results to quantitative predictions of service life. Nevertheless such tests can be made to indicate with considerable reliability whether one wood is likely to last longer than another in the same situation and whether the difference is likely to be large or small. Also, they are particularly well suited to disclose rapidly trends of association between decay resistance and other measurable characters of the wood.

MATERIALS AND METHODS

SOURCE AND KINDS OF TEST TREES

The seven species of oak tested follow:

White oak group:
  White oak (*Quercus alba* L.).
  Oregon white oak (*Q. garryana* Doug. ex Hook.).
  Chestnut oak (*Q. montana* Willd.).
  Swamp chestnut oak (*Q. prinus* L.; syn., *Q. michauxii* Nutt.).
Red oak group:
  Northern red oak (*Q. borealis* Michx.).
  Scarlet oak (*Q. coccinea* Muench.).
  Black oak (*Q. velutina* Lam.).

The location of the trees in the nine different regions that were considered is shown in figure 1.

To minimize any variation in decay resistance that might be caused by differences in tree size, dominants and codominants within a range of diameters at breast height (d. b. h.) from 15 to 18 inches were used exclusively in all phases of the study except the phase dealing specifically with the relation of decay resistance to tree size. With a few exceptions six to eight trees of a species were sampled in each locality.

In addition to the trees selected in localities shown in figure 1, 32 white oak trees having a wide range of diameters were sampled in a locality near Madison, Wis.; these furnished all the information that was gathered relative to decay resistance and tree size.

TEST SPECIMENS

The test samples were removed from the trees in the form of increment-borer cores, 0.21 inch in diameter, and as small blocks. The cores were relied upon for evidence of differences in decay resistance among trees and among localities and regions and were taken from all

FIGURE 1.—General regions in which the test oak trees were located: C, Central; MR, upper Mississippi River Valley; NA, northern Appalachian; NE, northeast coast; P, Pacific coast; PP, southeast Piedmont Plateau; S, southern; SA, southern Appalachian; SE, southeast coast.   The numerals following the region symbols designate the well-separated localities.

trees.   The blocks were used for determinations of trends of decay resistance in different parts of individual trees and were taken from only 17 of the trees.

Cores were used in most of the tests because they could be rapidly obtained from a large number of standing trees without serious injury to them.   Although only about one-tenth the size of the blocks, they appeared from correlation and variance analyses to give as reliable results if they were weighed more accurately.   Six cores were taken from each tree at breast height, three from one side and three from the side directly opposite.   The sapwood was eliminated and the outermost 2 inches of heartwood was tested.   The sapwood was identified or its identity confirmed in all cases by means of a colorimetric pH test, as described in Forest Products Laboratory Technical Note 253 (*10*).[3]

The blocks, 1 by 1 by ¼ inch in size with the short axis parallel with the grain, were obtained from felled trees.   They were sawed out consecutively across an entire diameter located at two different heights in the trunk, one at breast height and the other near the upper limit of the merchantable part of the trunk.

Most of the specimens were collected in February or March, when the trees were dormant in all localities except Louisiana and Texas. In the specimens from these States there was no evidence that the postdormant condition was a material factor of the decay resistance. Samples were taken only from trees having characteristic fruit and bark as described for the species.   The identity of the samples of

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[3] Italic numbers in parentheses refer to Literature Cited, p. 152.

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black oak was confirmed by observing the bark in the laboratory under ultraviolet light. The inner bark typically fluoresces with a blood-red color not observed in the other species.

TESTING PROCEDURE

Decay resistance was ascertained by exposing the cores and blocks to pure cultures of selected decay fungi. This was done in 6-ounce glass bottles of the French-square type, each containing 25 ml. of malt-agar nutrient medium.[4] The fungus was first allowed to cover the surface of the medium. A sterile V-shaped glass rod, 4 mm. in diameter, was then laid on the center of the fungus mat; this served as the support for the single test specimen introduced directly afterward into each bottle. Before the specimens were tested, they were given a partial sterilization treatment by steaming them while air-dry in glass bottles for 20 minutes at 212° F. Such treatment of air-dry wood probably affects its decay resistance very little. The test units were incubated for 4 months at 80°.

The extent of decay was measured by the percentage loss in dry weight of wood. Both before and after they were tested, the specimens were dried to constant weight in equilibrium with a relative humidity of 30 percent and a temperature of 80° F. The percentage losses in weight were determined on the basis of these weights, thus giving a result essentially on an oven-dry basis but with the advantage that no risk of altering the decay resistance by actual oven drying was entailed.

Initially, three different decay fungi were used in considering all variables except diameter. These were *Poria monticola* Murr. (Madison 698),[5] a very destructive species causing brown rot and perhaps one of the most prevalent fungi attacking pine, Douglas-fir, and oak boat timbers (*4*); *Lenzites trabea* Fr. (Madison 617), a brown rotter that attacks a variety of woods; and *Stereum frustulosum* Fr. (R. P. 56461–R), which produces a white pocket rot in dead oak timber and logs and which was the only white rot fungus frequently isolated from boat timbers.

Two cores from opposite sides of each tree and two blocks from each position sampled in the felled trees were tested against each fungus, thus making a total of six independent measures of decay resistance per tree or position in a tree.

The relation of white oak decay resistance to d. b. h. was ascertained about 3 years after the other phases of the study had been completed. By that time it had become apparent in supplementary observations that, although brown rot fungi are common in the decay of oak posts, ties, and structural wood, white rot fungi are probably fully as important generally. For example, in 34 black oak fence posts near Madison, Wis., about 20 percent of the heartwood decays were mixed brown and white rots, 24 percent were brown rots, and 56 percent white rots. In a study of decay of oak ties and posts Roth (*6*) found that 3 of the 6 fungi responsible for most of the decay resulting from infections subsequent to felling of the trees were white rotters. Con-

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[4] Consisting of 2.5 percent of Trommer's diastasic malt extract and 1.5 percent of agar in distilled water.

[5] This fungus was originally called *Poria microspora* Overh. It was received from Ross W. Davidson, Division of Forest Pathology, as Wash. 106.

sequently, in the consideration of the d. b. h. variable only *Poria monticola* and *Stereum frustulosum* were retained of the original 3 fungi and with these was used *Polyporus versicolor* Fr. (Wash. 72074, or Madison 697), one of the most destructive, widespread, and versatile of the white rot fungi attacking hardwoods.

In order to determine whether the conclusions derived from use of the original three fungi would also be applicable to decay by strictly white rot fungi,[6] a repeat test was made on wood blocks (p. 130) For this purpose *Poria monticola*[7] and *Polyporus versicolor* were used again, with two additional white rotters—an unidentified species (Madison 4411–1) that had been isolated from decayed gum wood in an Army glider and Madison 517,[8] a species of uncertain classification that had been widely used over a long period for testing the toxicity of wood preservatives.

RESULTS

RELATIVE DECAY RESISTANCE OF THE TESTED OAK SPECIES

The relative resistance of the tested oak species to decay by the three fungi used in the initial testing is indicated in figure 2. Species having the largest percentage weight loss had, of course, the lowest resistance. The order of differences in resistance between the white oak and red oak groups is in line with general service experience. The differences in resistance shown for the four species of the white oak group were not anticipated, however, and it is apparent from these differences that species in the white oak group are by no means in a single class of decay resistance. Insofar as these results may be generally representative, white oak (*Quercus alba*) would appear to occupy a more or less intermediate position of resistance in the white oak group. On the average the chestnut oak was substantially more resistant than the white oak, and the swamp chestnut oak was considerably less resistant. The Oregon white oak, although represented by very few trees, appears to be at least as resistant to decay as the white oak.

The species of the red oak group (black, northern red, and scarlet) lost on the average about four times as much weight by decay as did the chestnut oak and more than twice as much as the white oak. Differences found among the three species of the red oak group were slight and of doubtful practical significance.

Although the foregoing general observations, which were based on the average results given by all three test fungi, are probably representative of differences among the species to be expected commonly, it should be noted that the differences in resistance among the species

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[6] Inasmuch as *Stereum frustulosum* produces a white pocket rot, it cannot be considered as a white rot fungus in the strictest sense. Moreover, it has been reported as failing to give the typical white-rotter reaction on gallic or tannic acid medium (*3*).

[7] Although *Poria monticola* had been used in the first tests, there was no certainty that some changes in the decay resistance of the wood might not have taken place during the subsequent 3-year period. Actually, the results obtained with this fungus in the first and the repeat test were substantially alike (correlation coefficient=0.88).

[8] This fungus has been listed in previous reports from this laboratory as *Fomes annosus* (Fr.) Cke., but now it has been found by several investigators to be culturally more like *Polyporus tulipiferus* (Schw.) Overh.

in the white oak group to the individual fungi were not altogether similar. The relative resistance of the respective species to the brown rot fungus *Poria monticola* was much like that indicated by the average results. On the other hand, the differences in resistance to the second brown rot fungus, *Lenzites trabea*, although the most marked of all between the red and the white oak group, were small and of doubtful significance among the species of the white oak group. Findings in other tests similarly have indicated *L. trabea* to be capable of attacking rather strongly hardwoods of moderate to low resistance but only weakly those of greater resistance. The white pocket rot fungus
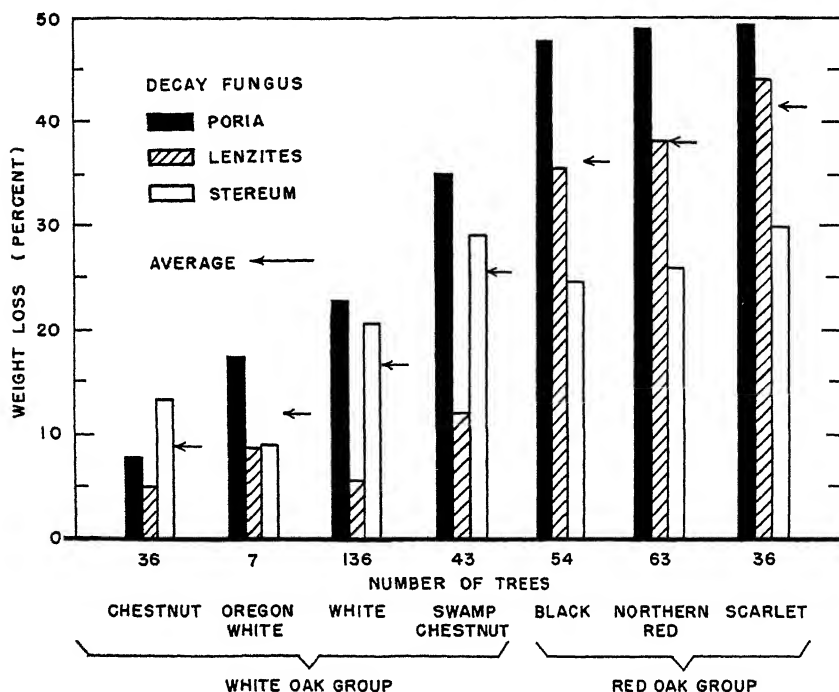
FIGURE 2.—Average weight loss caused by decay in cores from trees of the different oak species. Species with largest percentage of weight loss had least decay resistance.

Stereum frustulosum, which is a specialized inhabitant of oak wood, decayed the species of the white oak group almost as rapidly on the whole as *P. monticola;* this indicated comparable differences in resistance among those species. *S. frustulosum* decayed the red oak species more slowly than either of the other two fungi, however, with the result that the difference in resistance to it between the red and the white oak group was smaller; only the chestnut and Oregon white oaks demonstrated resistance to *S. frustulosum* substantially superior to that of the red oak group.

The tests on wood from different positions in the tree, described on page 148, furnish limited additional data on the resistance of the chestnut, white, swamp chestnut, and northern red oaks, in this case to

the three typical white rot fungi, *Polyporus versicolor*, Madison 517, and Madison 4411-1. The species differences in weight loss caused by these fungi were on the average smaller than those caused by the other fungi, mainly because of a slower general attack by Madison 517 and Madison 4411-1 and the fact that, although *P. versicolor* decayed the white oak species more rapidly than any of the other fungi, the differences in resistance to this fungus were not correspondingly larger. Of greater significance, however, is the fact that the relative resistance to the three fungi exhibited by the outer heartwood (representing the major volume of heartwood) of the species was indicated to be very much like that denoted by the average weight losses shown in figure 2. The remainder of the heartwood was characterized by a reversal in the order of resistance between the northern red and the swamp chestnut oak and between the white and the chestnut oak.

VARIABILITY IN DECAY RESISTANCE AMONG TREES OF THE SAME SPECIES

Some further light is shed on the species differences by observing the variability in decay resistance among the trees of the respective species as shown in figure 3. This figure is based on smoothed cumulative-frequency curves of the weight loss. Essentially, each species diagram is a double frequency histogram having unequal class intervals. The asymmetry of a number of the diagrams undoubtedly can be
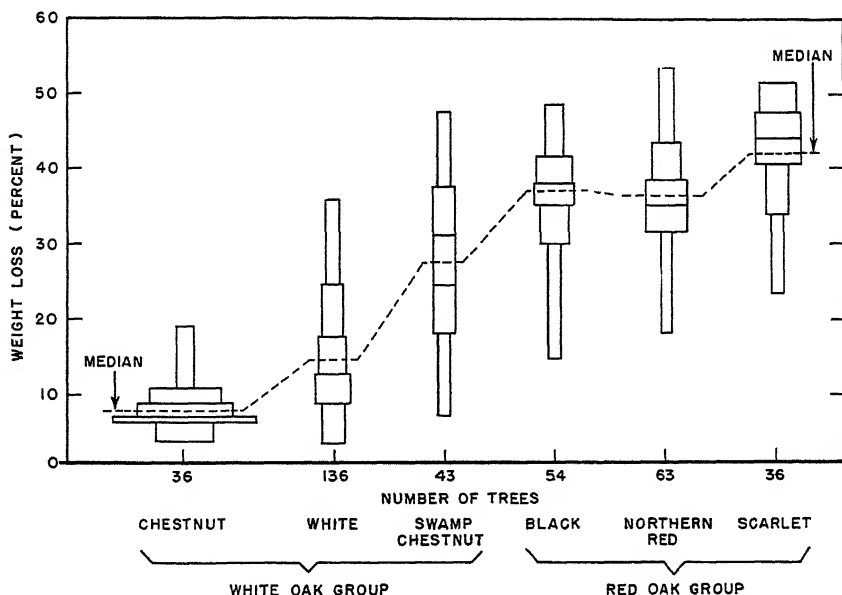
FIGURE 3.—Distribution diagrams for weight loss by trees in the different oak species, based on averages for *Lenzites trabea*, *Poria monticola*, and *Stereum frustulosum*. All diagrams have the same total area, representing 100 percent of the trees of the different species, and each of the 5 segments in a diagram has the same area (20 percent of the total) and represents 20 percent of the trees as arranged in order of weight loss. Thus the percentage of trees of a given species falling within a particular weight-loss range can be estimated from the percentage area occupied by the corresponding portion of the species diagram.

ascribed partly to the comparatively small number of trees sampled. It is also evident, however, that some of the "bunching" of trees, which was most marked with chestnut and scarlet oaks, is a reflection of the restrictions on weight loss imposed by zero and the upper limits of weight loss. The upper limit for brown rot fungi, as indicated in previous tests by the writers and in tests by Findlay (5), appears to be near 60 to 65 percent.

Perhaps the most striking information brought out by figure 3 is the fact that the variability in resistance among trees of a particular species may be comparatively large.[9] Consequently, although the average resistance of one species may differ substantially from that of another, a considerable proportion of the trees of the two species may have wood in the same resistance class. For example, although the wood of the black oak trees lost on the average more than twice as much weight as that of the white oak (*Quercus alba*) trees, about 50 percent of the white oak trees and about 40 percent of the black oak fell within the same range of weight losses (15 to 35 percent). This evidence in itself might well account for conspicuous differences in the service life of oak wood of the same species. Moreover, it reveals the possibility of growing oak wood of greatly improved decay resistance by propagating strains of superior resistance. If vegetative propagation of oak ultimately becomes practicable or if seedling progenies inherit a sufficient portion of parental resistance, strains of white oak or swamp chestnut oak might be selected, for example, with two or even three times the average decay resistance of those species. Not only would a higher average resistance thus be provided, but also a more uniform and therefore a more predictable service life would result. The weight-loss distribution diagram for the chestnut oak (fig. 3) would seem to be a conservative illustration of what might be accomplished along this line.

Another possibility would be selection of red oak species with heartwood resistance equal to that of the average white oak, which, with the capacity of the red oaks to take preservative treatment, would give ties and structural timbers combining strength and decay resistance to a high degree.

Not only resistance to decay in service but also resistance of standing trees to heart rot might be improved by such selection. Judging from the findings in a recent study of the wood of black locust (*Robinia pseudoacacia* L.),[10] the decay resistance may be as characteristic and permanent a feature of a given strain of timber as anything else about it. Stands of oak once established reproduce more often by sprouts after cutting than from seed, thus tending to perpetuate the established strains.

No clear difference was found between the decay resistance of white oak (*Quercus alba*) wood and wood from trees of the same species

[9] The ranges of weight loss illustrated in figure 3 are somewhat larger than those representative of the actual variation in decay resistance. That is because the weight losses also include the experimental error of determining them. Increases from this source, however, are probably no larger than, if as large as, the increases that would be had purely as a result of increasing the sampling basis.

[10] SCHEFFER, T. C., and HOPP, H. THE DECAY RESISTANCE OF BLACK LOCUST HEARTWOOD. (Unpublished manuscript.)

sometimes referred to in the Northeast as "yellow-bark oak." Likewise, no difference was found between northern red oak wood and so-called "gray oak" wood of this same species.

LOCALITY AND REGIONAL DIFFERENCES IN DECAY RESISTANCE OF THE TESTED OAK SPECIES

The over-all variability just observed in the decay resistance among trees of a given oak species does not appear to consist simply of more or less uniform differences among the trees everywhere. Rather it appears to be partially contributed by differences in average levels of resistance of wood from the different localities and regions. Many such differences were very marked. For example, in table 1 it may be noted that the average weight loss produced by *Poria monticola* in white oak wood from locality 1 of the upper Mississippi River Valley region was 36.3 percent, whereas that in wood from locality 3 of the same region was only 5.9 percent. Similarly, the average weight loss caused by *P. monticola* in white oak from this region as a whole was 19.4 percent, whereas that in wood from the northeast coast was 31.9 percent. Smaller but nevertheless considerable differences appear in the corresponding averages for decay by the other two fungi.

Two questions raised by these findings are (1) whether such locality and regional differences might be purely a random result, the main features of which would not necessarily be obtained if other sets of trees were sampled on the same areas and (2) whether these differences if they are real are of genetic origin or are attributable to differences in conditions under which the trees were grown. A general answer to the first question may be had by treating the data statistically according to the method of analysis of variance. Terminal computations needed for this analysis are given in table 2.

If the differences in weight loss between cores from opposite sides of the trees (section D) are taken as the nearest measure of experimental error, it is apparent from the number of significantly larger variances of section C (denoted by significant *F* values) that there were real differences in decay resistance among white oak trees even in the same locality. Inasmuch as the trees in any one locality were of approximately the same size and age and were grown in practically the same general environment, this may be construed as an indication of genetic differences in resistance. A similar and rather general variation in resistance among trees within localities is indicated in the other species considered in table 2; it was not so pronounced as with the white oak, however, and was statistically significant for considerably fewer of the regions individually.

In the same way the variation in resistance among trees within localities can be used to test the variation in average resistance of wood from the different localities of a region (section B). Twelve of the twenty-seven locality variances for individual regions in section B were significantly larger than the corresponding tree variances in section C; thus they were larger than might reasonably be attributed to random differences among the sampled trees. Although the number of localities considered in each region was too small to permit appraisal

TABLE 1.—*Average weight loss caused by decay in wood from various oak species, regions, and localities*

Region and locality from which test samples were obtained	Decay caused by *Poria monticola* in—							Decay caused by *Lenzites trabea* in—						
	White oak	Oregon white oak	Chest-nut oak	Swamp chest-nut oak	North-ern red oak	Black oak	Scarlet oak	White oak	Oregon white oak	Chest-nut oak	Swamp chest-nut oak	North-ern red oak	Black oak	Scarlet oak
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Upper Mississippi River Valley (MR):														
1	36.3				45.0	38.2		6.4				33.7	30.6	
2	15.9				48.0	45.0		3.7				34.2	36.4	
3	5.9				48.5	40.0		4.0				25.9	19.8	
Average	19.4				47.2	41.1		4.7				31.3	29.0	
Central (C):														
1	23.4							7.0						
2	18.3							3.9						
3	20.2							4.4						
4	11.0							4.1						
Average	17.2							4.7						
Northeast coast (NE):														
1	12.2				54.4	55.7	46.3	5.7				45.6	42.9	34.6
2	35.6				51.7	52.7	44.2	7.1				37.5	39.6	46.3
3	44.6				54.3			10.4				53.7		
4			14.8							4.5				
Average	31.9		14.8		53.5	54.2	45.3	7.9		4.5		46.0	41.2	40.5
Northern Appalachian (NA):														
1	39.1				44.1			7.1				31.4		
2			7.9							4.1				
3			4.3							4.4				
Average	39.1		6.1		44.1			7.1		4.3		31.4		
Southern Appalachian (SA):														
1	23.9							7.2						
2	29.7		8.5		42.3	50.5	53.5	4.5		6.3		38.6	35.8	53.1
3	21.2		6.2		52.6	48.5	56.5	4.6		5.2		36.3	39.9	42.3
4	15.9					54.0	53.2	4.3					43.0	52.8
5														
Average	22.3		7.4		47.4	51.0	54.4	4.6		5.7		37.5	39.6	49.4

Locality														
Southeast Piedmont Plateau (PP):														
1	30.7	33.1	42.9		5.0			43.3	45.0	48.3		4.2		
2														
Average	30.7	33.1	42.9		5.0			43.3	45.0	48.3		4.2		
Southeast coast (SE):														
1				6.2							19.8			
2				17.1							41.7			
3				12.9							43.8			
4				8.0							26.8			
Average				11.1			4.6				33.0			14.7
Southern (S):														
1				17.0		8.9	7.6				42.4		19.9	17.0
2				25.8		8.7	5.5				51.2		11.1	31.3
Average				18.2		8.8	7.2				43.7		17.4	19.9
Pacific coast (P):														
1													19.9	17.4
2													11.1	
Average													17.4	17.4
Species average	44.3	35.7	38.5	12.2	4.9	8.8	5.6	49.5	47.7	49.1	34.8	7.7	17.4	22.7

TABLE 1.—*Average weight loss caused by decay in wood from various oak species, regions, and localities*—Continued

Region and locality from which test samples were obtained	Decay caused by *Stereum frustulosum* in—							Average decay caused by all fungi in—						
	White oak	Oregon white oak	Chestnut oak	Swamp chestnut oak	Northern red oak	Black oak	Scarlet oak	White oak	Oregon white oak	Chestnut oak	Swamp chestnut oak	Northern red oak	Black oak	Scarlet oak
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Upper Mississippi River Valley (MR):														
1	24.7				31.1	17.6	17.9	22.5				36.6	28.8	32.9
2	14.6				21.3	23.3	31.9	11.4				34.5	34.9	40.8
3	9.1				16.2	13.8		6.3				30.2	24.5	
Average	16.2				22.9	18.2	24.9	13.4				33.8	29.4	36.9
Central (C):														
1	20.9							17.1						
2	22.7							15.0						
3	20.0							14.9						
4	20.6							11.9						
Average	21.0							14.3						
Northeast coast (NE):														
1	18.1				32.2	26.2		12.0				44.1	41.6	
2	23.2				19.4	28.0		22.0				36.2	40.1	
3	27.7				29.6			27.6				45.9		
4			11.8							10.4				
Average	23.4		11.8		27.0	27.1		21.1		10.4		42.2	40.8	
Northern Appalachian (NA):														
1	22.0				25.0			22.7				33.5		
2			20.8							10.9				
3			11.4							6.7				
Average	22.0		16.1		25.0			22.7		8.8		33.5		
Southern Appalachian (SA):														
1	33.6							21.6						
2	22.9		8.5		25.8	18.8	28.9	19.0		7.8		35.6	35.0	45.2
3	23.5		15.3		27.4	30.4	32.2	16.4		8.9		38.8	39.6	43.7
4	20.7					33.8	34.4	13.6					43.6	46.8
5														
Average	22.8		11.9		26.6	27.7	31.8	16.6		8.3		37.2	39.4	45.2

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14
Southeast Piedmont Plateau (PP):														
1														
2	36.3	36.5	41.1		7.3			34.9	31.5	32.1		12.6		
Average	36.3	36.5	41.1		7.3			34.9	31.5	32.1		12.6		
Southeast coast (SE):														
1				15.5							20.6			
2				29.8							30.6			
3				29.9							33.1			
4				21.1			11.9				28.4			16.4
Average				24.1			11.9				28.2			16.4
Southern (S):														
1				30.7			14.9				32.7			20.2
2				37.3			20.7				34.8			25.2
Average				31.6			16.1				33.0			21.2
Pacific coast (P):														
1						12.7							9.3	
2						9.5							8.7	
Average						11.8							9.1	
Species average	41.3	36.1	37.9	25.3	8.7	11.8	16.3	30.0	24.8	26.0	29.0	13.4	9.1	20.7

TABLE 2.— *Tree, locality, and regional differences in decay resistance indicated by analysis of variance of the weight losses*

Source of weight-loss variation	White oak decay caused by—						Chestnut oak decay caused by—					
	Poria			Stereum			Poria			Stereum		
	Degrees of freedom	Variance	Variance ratio (F)[1]	Degrees of freedom	Variance	Variance ratio (F)[1]	Degrees of freedom	Variance	Variance ratio (F)[1]	Degrees of freedom	Variance	Variance ratio (F)[1]
A. Among means of regions	6	1,039.9	1.1	6	161.5	1.1	3	135.7	5.0	3	44.4	0.2
B. Among means of localities within regions:												
All regions	11	959.4	3.1**	11	149.2	3.2**	2	27.0	.6	2	201.9	8.7**
In region MR[2]	2	1,914.0	10.0**	2	498.6	11.3**						
In region C	3	269.0	.7	3	11.2	.2						
In region NE	2	2,404.0	11.9**	2	206.8	5.0*						
In region NA							1	38.9	.9	1	265.1	8.4*
In region SA	3	261.8	.6	3	52.1	1.4	1	15.2	.4	1	138.7	6.5*
In region SE												
In region S	1	324.9	.8	1	41.0	1.7						
C. Among means of trees within localities:												
All localities	118	308.7	5.3**	118	47.0	4.7**	30	48.0	2.0*	30	23.2	1.8*
In region MR	21	191.1	4.4**	21	44.1	10.5**						
In region C	31	378.7	6.1**	31	66.2	4.2**						
In region NE	23	201.6	2.2*	23	41.5	4.6**						
In region NA	7	216.8	3.1	7	47.9	3.2	5	133.0	3.4	5	17.0	1.2
In region SA	21	441.4	10.9**	21	37.3	3.8**	10	42.9	3.6*	10	31.5	2.3
In region PP							10	34.0	.8	10	21.3	1.2
In region SE	7	292.9	10.1**	7	41.9	6.5**	5	.4	.3	5	16.8	8.4*
In region S	8	400.4	10.5**	8	24.7	4.8*						
D. Between cores from opposite sides of trees:												
All trees	129	58.0		134	9.9		36	24.2		36	13.0	
In region MR	20	43.8		24	4.2							
In region C	33	62.1		33	15.6							
In region NE	26	92.9		26	9.0							
In region NA	8	69.1		8	15.1		6	39.7		6	14.0	
In region SA	24	40.3		25	9.9		12	11.8		12	13.8	
In region PP							12	40.2		12	17.2	
In region SE	8	29.1		8	6.4		6	1.2		6	2.0	
In region S	10	38.2		10	5.1							

Source of weight-loss variation	Swamp chestnut oak decay caused by—						Red oak decay caused by—								
	Poria			*Stereum*			*Poria*			*Stereum*			*Lenzites*		
	Degrees of freedom	Variance	Variance ratio (F)[1]	Degrees of freedom	Variance	Variance ratio (F)[1]	Degrees of freedom	Variance	Variance ratio (F)[1]	Degrees of freedom	Variance	Variance ratio (F)[1]	Degrees of freedom	Variance	Variance ratio (F)[1]
A. Among means of regions	1	667.5	0 7	1	133 8	0 7	4	162 4	2 1	4	104.5	0 4	4	638 6	2 5
B. Among means of localities within regions:															
All regions	4	923 7	2 8*	4	198 3	3 0*	5	78.9	1.7	5	265.3	5.8**	5	252 7	1.5
In region MR[2]							2	21.6	4	2	349.3	17.3**	2	130 8	8
In region C															
In region NE															
In region NA															
In region SA	3	1,209 7	3 4*	3	263 2	4 1*	2	17.0	1 0	2	310 5	4.7*	2	492.7	4 9*
In region SE	1	65 6	.5	1	3 8	0	1	317 2	5.8*	1	6 8	.1	1	16 6	1
In region S															
C. Among means of trees within localities:															
All localities	37	325.0	4.9**	37	65.7	9.7**	53	46 7	1.4	53	45.8	2 1**	53	167 8	2 3**
In region MR							15	48 0	1 2	15	20 2	9	15	156.0	3 1*
In region C															
In region NE							18	16 4	1 1	18	66.1	3.3**	18	99 7	2 1
In region NA							5	160 4	7 1*	5	28.1	1 6	5	249.6	2 8
In region PP	32	354 1	5 0**	32	64.0	8.9**	10	54 9	8	10	52 9	1 8	10	253.8	2 0
In region SE	5	142 6	3 4	5	76 6	16.6**	5	23 4	6	5	53 1	2 5	5	194 6	1 8
In region S															
D. Between cores from opposite sides of trees:															
All trees	42	66.1		43	6 8		61	33 8		63	22 0		63	74 2	
In region MR							16	39.8		18	21.6		18	49.5	
In region C															
In region NE							21	14 2		21	20.2		21	48 5	
In region NA							6	22.6		6	17.1		6	89 7	
In region SA															
In region PP	35	71 0		36	7.2		12	64 5		12	28.6		12	128.7	
In region SE	7	41 5		7	4 6		6	36.2		6	20 9		6	110 4	
In region S															

*Statistically significant difference at the 5-percent level.
**Statistically significant difference at the 1-percent level.
[1] Obtained by dividing the variance by the corresponding variance below it in the table. For example, variances in group A are tested by the corresponding variances in group B, those in group B by corresponding ones in group C, etc.
[2] Region symbols explained in legend for figure 1.

of the situation in general, this result suggests that to a limited extent at least there tends to be a closer genetic relation among trees of a given oak species in the same locality than there is among the trees of different localities. This inference is valid, of course, only if environmental or other extraneous factors had no prominent influence on the results.

Regional differences in the average decay resistance of a particular species of oak (section A) are indicated by the variance ratios to have been in no case larger than might be expected among localities of comparable size in a single region. It may be noted in this connection that for testing the regional differences the pooled variances of section B, representing the average among-localities variances for all regions, were used. In spite of the marked differences in magnitude among some of the variances that were pooled there was no evidence in tests of variance homogeneity (9) that any of these differences were sufficiently large to preclude pooling. It may be further noted that even when tested by the individual corresponding variances of smallest magnitude in section B, only two of the nine regional variances (section A) were indicated to be statistically significant.

Three practical conclusions from table 2 and the foregoing variance analysis are suggested:

(1) Oak trees of the same species and approximate size from the same wood lot or locality commonly will have marked differences in decay resistance.

(2) Wood of the same oak species from one locality often averages consistently higher or lower in decay resistance than that from another locality in the same region.

(3) Oak wood from one region as a whole is not likely to be appreciably more resistant than wood of the same species from another region; occasional impressions to the contrary are probably gained from wood taken from comparatively limited areas, such as localities, and thus not adequately representing whole regions.

Regarding the question whether these tree and locality differences are of genetic origin, no reliable evidence could be found of a relation between the decay resistance of individual trees in a locality and differences in their immediate environment or in their most recent growth characteristics as measured by ring counts in the outer heartwood and the sapwood, respectively, and by the width of the sapwood. However, a slight relation between ring count and width of sapwood and locality differences in average decay resistance was indicated. Among localities the resistance tended to be less as the growth rings in the outer heartwood and in the sapwood were narrower and as the sapwood was narrower. By far the best evidence of this was shown by *Poria monticola* decay in the white oak, but the correlation coefficients characterizing the relation among localities of weight loss to ring width in the outer heartwood and the sapwood and to sapwood thickness in this species were only -0.47, -0.56, and -0.58, respectively, (based on 18, 17, and 17 localities, respectively). At most, therefore, only about one-third of the variance among localities in the resistance of the white oak to decay by *P. monticola* might be explained by differences in any of these growth characters, and much less of the variance in white oak resistance to the other two fungi or of the

variance in resistance among localities of the other oak species could be attributed to differences in these characters.

To check further on the possibility of environment as a factor of locality differences in decay resistance, computations were made of the correlation between locality averages of weight loss for pairs of species that came from the same localities. The assumption was that if environment were an appreciable factor this would be indicated by a tendency for the decay resistance of the different species to vary in similar order from locality to locality. An over-all tendency of this sort is denoted by the correlation coefficients shown in table 3. But here, also, the generally low values of the coefficients (which were individually significant statistically in only one instance) indicate that environmental differences among the localities might explain only a comparatively small part of the differences. From this and the preceding evidence, therefore, it is concluded that at least a large share of the observed differences in resistance within species is probably attributable to strain differences.

TABLE 3.—*Correlation between locality averages of weight loss in different oak species from the same localities* [1]

Species compared	Localities	Correlation coefficient (r)
Northern red oak and—	*Number*	
White oak	9	0.41
Black oak	7	.77*
White oak and—		
Swamp chestnut oak	3	.96
Black oak	7	.24
Scarlet oak	4	.50
Scarlet oak and—		
Chestnut oak	3	.62
Northern red oak	4	−.24
Black oak and—		
Chestnut oak	3	.80
Scarlet oak	6	.02

*Statistically significant at the 5-percent level.
[1] Averages based on weight loss caused by *Poria monticola*, *Lenzites trabea*, and *Stereum frustulosum*. (See table 1.)

RELATION OF DECAY RESISTANCE TO SIZE OF TREE

Decay resistance in relation to size of tree was determined only for white oak on the assumption that any tendencies exhibited would probably apply, in some degree at least, to the other species. Data were obtained on increment-core test samples from 32 trees in a single locality. It is apparent from the plotted results (fig. 4) that there was a rather marked tendency for the decay resistance of the outer heartwood to increase with size of tree. This was true with respect to all three of the test fungi and, as indicated by the correlation coefficients, with nearly equal prominence.

It should be noted that the trees on which this diameter-relation study was made were in an approximately even-aged, closed stand in which some of the trees were suppressed, so that large differences in diameter occurred when age was fairly constant. But if oak follows the same general pattern as has been found for black locust,[11] much the same relation between outer heartwood resistance and d. b. h.

[11] See footnote 10, p. 136.
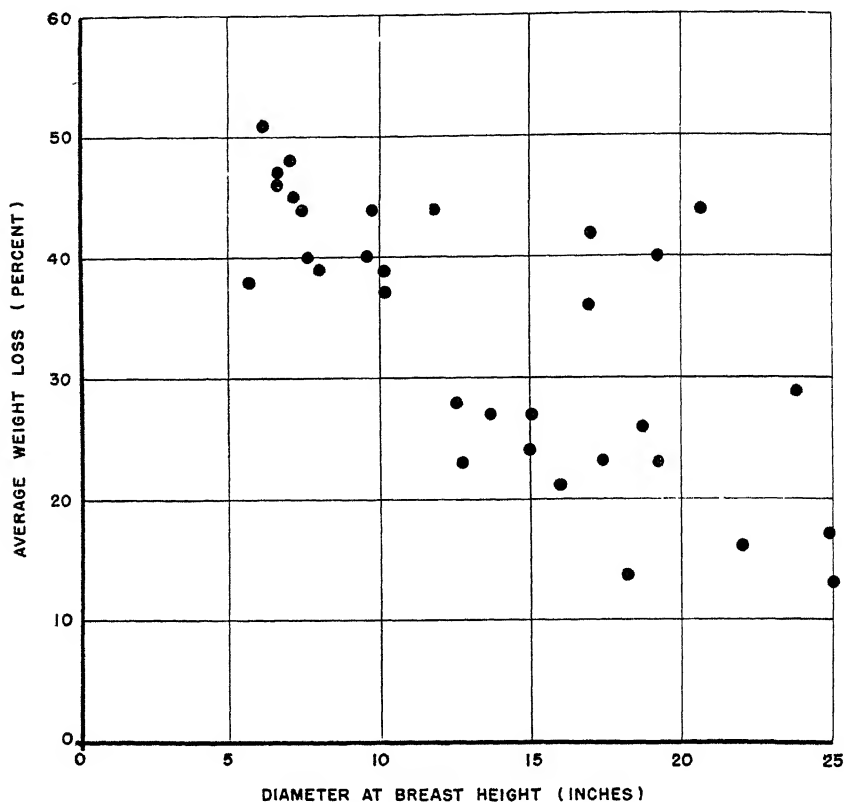
FIGURE 4.—Relation between the diameter of white oak (*Quercus alba*) trees in a single stand and the weight loss caused by decay in wood from the outer heartwood. (Each point represents the average weight loss caused by *Poria monticola*, *Polyporus versicolor*, and *Stereum frustulosum* in the six cores from each tree. The correlation coefficients for d. b. h. and weight loss caused by these fungi individually were −0.72, −0.75, and −0.73, respectively.)

might be expected in open-grown trees of different ages as well as sizes.

VARIABILITY IN DECAY RESISTANCE WITHIN INDIVIDUAL TREES

As stated, ratings of the decay resistance of the respective trees were based on increment cores taken from the lower trunk in the outer 2 inches of heartwood. Such restricted sampling obviously could lead to erroneous conclusions if this part of the trees did not represent reasonably well the entire merchantable part of the trunk. Partly as a check on this point, a number of trees were tested by block samples taken consecutively through the trunk. It also was desired to find out whether there might be large enough differences in decay resistance among various parts of the trunk to warrant consideration when using wood from large trees. The results of this phase of the testing, with the three fungi used first, are summarized in figure 5. The trees sampled for this part of the study, like those sampled for species and tree differences, were in the 15- to 18-inch diameter class; moreover, the individual trees of each species came from different, widely separated localities.

The outer heartwood of all four species represented was more resistant to decay by the three fungi used in determining species and tree differences than was the inner heartwood in both the lower and the top portion of the trunks. Moreover, the change in resistance from the outer to the inner heartwood, although tending to be irregular in individual trees, was on the average progressive. With respect to the major volume of wood in the trunk,[12] these over-all trends did not differ greatly with the different species. The inner, lower-trunk portion of the northern red oaks, which failed to show the same trend, represented considerably less than half the heartwood volume of the trees. Consequently, much the same relative order of decay resistance of the species dealt with (fig. 2) presumably would have been indicated had the heartwood been sampled more completely.

In the larger logs, as indicated by the lower-trunk curves of figure 5, the differences in decay resistance between the inner and the outer heartwood clearly were as great as the differences between some of the oak species. Markedly superior durability is not to be expected
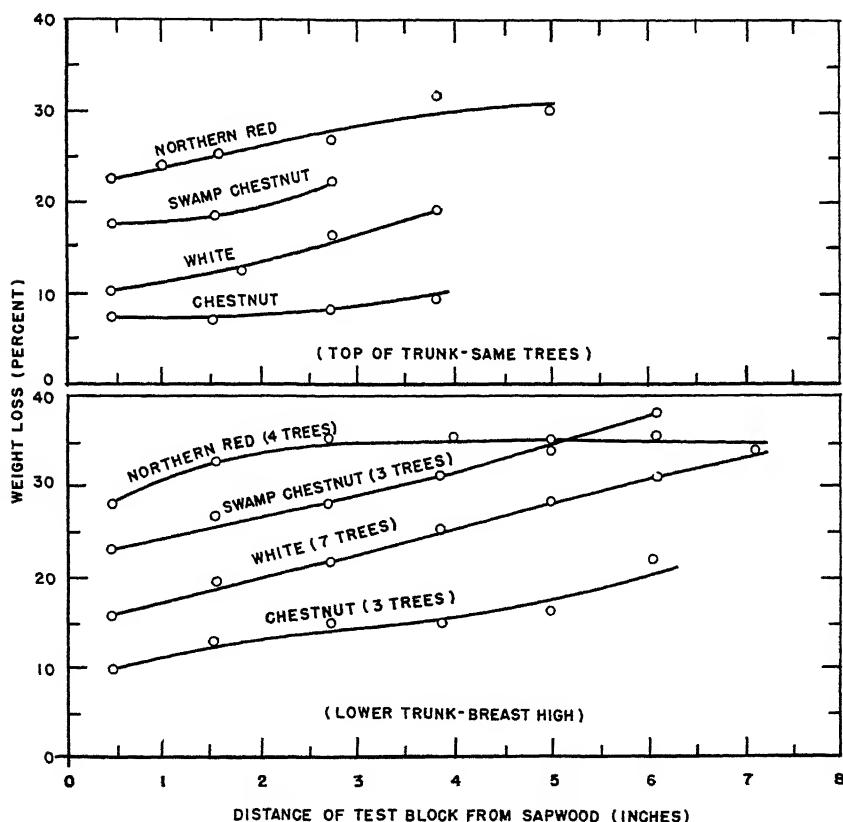
FIGURE 5.—Weight loss in blocks from different parts of individual oak trees. (Based on averages for the indicated number of trees and decay caused by *Poria monticola, Lenzites trabea,* and *Stereum frustulosum.*)

[12] The outer half of the heartwood radius represents approximately three-fourths of the total heartwood volume.

in the central heartwood of large logs, even of the more decay-resistant white oaks. Similar radial decreases in decay resistance from the outer to the inner heartwood have been reported for a number of species other than oak (*1, 2, 7, 8*).[13]

It should be kept in mind that this variability in decay resistance within individual trees also may be more representative for some fungi than for others. Only slight or no radial trends were found in the average weight loss caused by the three white rot fungi, *Polyporus versicolor*, Madison 517, and Madison 4411–1 (fig. 6). In white oak

R- NORTHERN RED   W- WHITE
S- SWAMP CHESTNUT   C - CHESTNUT

DISTANCE OF TEST BLOCK FROM SAPWOOD (INCHES)
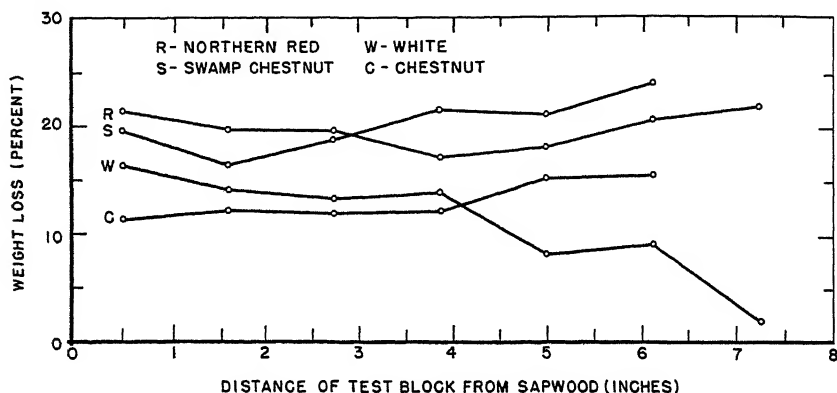
FIGURE 6.—Average weight losses caused by three white rot fungi, *Polyporus versicolor*, Madison 517, and Madison 4411–1, in oak blocks from the basal portion of the same trees and the same radial positions as represented in figure 5.

the trend was the reverse of that shown in figure 5, reflecting mainly the influence of Madison 517 and Madison 4411–1. The average radial trend of resistance to *Polyporus versicolor*, the most destructive of the three fungi, was from higher resistance in the outer heartwood to lower resistance in the central heartwood in all the species except northern red oak, in which no trend was apparent. No consistent radial trends were apparent in the upper trunks in tests made with these fungi.

In considering the differences between figures 5 and 6, it should be recalled (pp. 133 and 134) that the wood was 3 years older when tested with the fungi represented in figure 6 than when tested with those represented in figure 5. It is possible, therefore, that changes in the fungus-inhibiting properties of the wood, which would make the radial trends in the two cases not strictly comparable, might have occurred during this interval. The only evidence on this point is had in the results obtained with *Poria monticola*, which was included in both series of tests. That the radial differences in resistance to at least this fungus had not changed markedly is indicated by the correlation coefficient (0.88) characterizing the relation between the *P. monticola* results in the two cases.

Figures 5 and 6 were also the basis of the supplementary estimate of species differences in resistance to white rot fungi (p. 133).

Rather marked differences in decay resistance vertically in the trunk also are revealed in figure 5. Taken at equal distances from the sapwood, the heartwood of the upper trunks was more resistant

[13] See footnote 10, p. 136.

to decay than that of the lower trunks.   Inasmuch as this difference occurred in the trees of all four species, it might reasonably be expected rather generally among the oaks.   Unlike the radial trends, however, it has not been found in the other woods studied.   The most durable heartwood of black locust, western red cedar, European larch, and redwood has been found in the basal portion of the trees *(1, 2, 8)*.[14]

Differences between the upper and the lower trunks in resistance to the three typical white rot fungi are indicated by the following average percentage weight losses for the top and bottom logs, respectively: Chestnut oak, 9.7 and 12.3; white oak, 12.3 and 14.2; swamp chestnut oak, 22.0 and 19.3; northern red oak, 12.4 and 20.5. Only for the swamp chestnut oak was the difference contrary to the results shown in figure 5.

The sapwood of all domestic species has generally been considered to have comparatively little resistance to decay.   The present findings for oak are quite in line with this belief; however, some sapwood differences paralleled the differences in heartwood.   In the tests represented by figure 7 the sapwood from the upper part of the trunk was indicated to be slightly more resistant than that from the lower trunks in three of the species; also, the sapwood of the white oaks was more resistant than that of the red oak.   Among the species of the white oak group, the resistance of the sapwood followed the same order as that of the heartwood except in the case of chestnut oak. It is interesting to observe here that the white oak (*Quercus alba*)
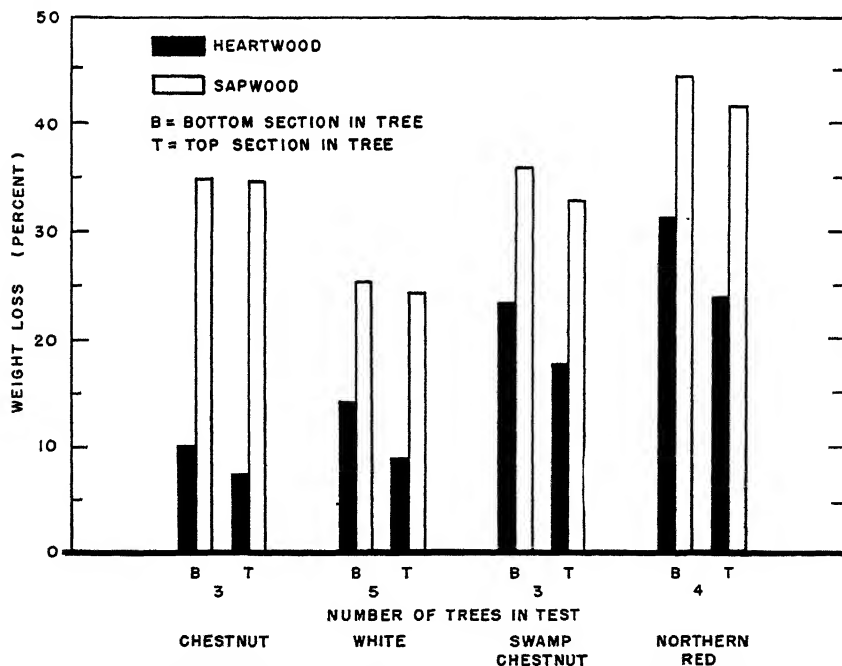
FIGURE 7.—Weight losses caused by decay in blocks from the outer heartwood and in adjacent sapwood blocks.   (Losses are averages for the indicated number of trees and for decay caused by *Poria monticola, Lenzites trabea*, and *Stereum frustulosum*.)

[14] See footnote 10, p. 136.

sapwood had about the same resistance as the red oak heartwood. However, little practical significance can be attached to the sapwood differences inasmuch as the level of resistance exhibited in all cases was comparatively low.

DECAY RESISTANCE AND THE SPECIFIC GRAVITY OF OAK WOOD

The relation of decay resistance to the specific gravity of the wood was not a specific subject of this study. Nevertheless, certain conclusions regarding it may be drawn from data already presented. First, it was found that the resistance of the outer heartwood of white oak trees tended to decrease as the width of growth rings narrowed. Such a relation among trees of the same general diameter has already been noted (p. 144), with a correlation coefficient of −0.47 for weight loss and width of rings in the heartwood. For trees of different sizes, represented in figure 4, the relation between percentage weight loss (caused by *Polyporus versicolor*) and ring width was characterized by a coefficient of −0.50. This evidence suggests that inasmuch as the specific gravity of hardwoods, especially the ring-porous species, also tends within limits to decrease as the ring width becomes narrower, the wood of lower specific gravity would decay somewhat more rapidly. However, the radial trends of decay resistance (figs. 5 and 6) give evidence contrary to this. Toward the center of the trees the specific gravity was progressively higher as a result of the more rapid growth when the trees were young, whereas the decay resistance was progressively lower or for much of the wood more or less uniform. It thus seems likely that the specific gravity of oak wood has little effect on its decay resistance, being largely overshadowed by other factors such as the location of the wood in the tree and the size of the tree.

SUMMARY AND GENERAL CONCLUSIONS

Laboratory decay tests were made on heartwood from 222 trees representing 4 commercial species in the white oak group and 153 trees representing 3 commercial species in the red oak group. The trees were located in 9 important oak-growing regions and in well-separated localities in each region.

Wood of the white oak group on the average was substantially more resistant to decay than that of the red oak group. This is in agreement with the general experience that species of the red oak group are not suited to uses involving considerable decay hazard.

Species of the white oak group were not uniformly resistant to decay, however, and some commercial distinction among them as to durability may be warranted. The most resistant of these species was chestnut oak; next in order of decreasing resistance were Oregon white oak, white oak (*Quercus alba*), and swamp chestnut oak.

There was no evidence of practical differences in resistance among the three species of the red oak group, namely, black oak, northern red oak, and scarlet oak.

Individual trees of the same species, of approximately the same size, and in the same locality differed markedly from one another in resistance. This was true of all four species examined, but especially so of white and swamp chestnut oaks. Practically and mathematically significant differences in average resistance also occurred among localities in the same region. Although considerable differences were also found among regions as a whole, they were generally no greater than

among localities within regions. The results thus indicate that it may be possible to obtain especially resistant oak wood by going to selected localities, but they do not support the belief that the oak wood of some entire regions is much superior in decay resistance to wood of the same species in other regions. Furthermore it is apparent that the use of white oak provides assurance of superior decay resistance in general but that some individual trees of this species and most of the trees in certain localities may have only moderate resistance.

This variability in resistance among trees appeared to be attributable more to genetic differences than to differences in the environment of the trees. The possibility presents itself, therefore, of selecting and propagating oak trees with wood having not only a longer but also a more uniform service-life expectancy.

The outer heartwood of white oak (*Quercus alba*) trees in the approximately even-aged stand studied for this relation was higher in decay resistance as the d. b. h. of the trees was larger.

In three species of the white oak group and in northern red oak the resistance to decay by two brown rot fungi and a white pocket rot fungus was progressively lower from the outermost heartwood to the pith. This trend occurred in both the basal and the upper portion of the trunks. Radial differences in resistance to decay by three other fungi, all white rotters, were generally smaller, and in white oak an opposite trend was indicated by two of the fungi. The differences with respect to the first three test fungi were large enough to account for very considerable differences in the service life of wood from different parts of large logs. In fact, some of the central heartwood of the white oak trees was no more resistant than that of northern red oak wood. This finding might be considered further justification for the common discrimination against boxed hearts (log centers) for such purposes as boat timbers.

In the same four species the heartwood in the upper trunks was, with one exception, more resistant to all the fungi than that in the lower trunks. In the swamp chestnut oak this difference was exhibited in resistance to the two brown rot fungi and the white pocket rot fungus, but not in resistance to the three typical white rotters. Heartwood of the top log might be expected, therefore, to remain serviceable as long as wood from other parts of an oak tree and probably substantially longer than central heartwood from the larger, basal log.

There was some association between the decay resistance of the sapwood and that of the heartwood. The sapwood of all species in which it was tested decayed comparatively rapidly, however, confirming the general experience that sapwood by and large is not durable.

From indirect comparisons, the specific gravity of the wood appeared to be no more than a minor factor in its decay resistance. Similarly, the rate of growth appears to have little possibility as a criterion of resistance.

The variability in decay resistance with locality, tree size, and position of the wood in the tree, as well as the uncorrelated differences among trees, indicates that comparisons of species for decay resistance may be unreliable unless many trees from numerous localities covering the range of the respective species are sampled and unless the trees are of approximately the same size (or of the sizes most

utilized) and the samples are taken from about the same place in each tree. It was indicated in the present study that if differences in the resistance of the outer heartwood also are suitably representative of differences in the resistance of the interior heartwood, as with the oaks, increment-borer cores from the outer 2 inches of heartwood provide satisfactory test specimens; their use makes it practicable to include many trees in the sample with minimum expense.

Cores must be taken at a uniform height; if all cores could be taken 8 or 10 feet from the ground, they would represent the tree as a whole somewhat better than if taken 4½ feet from the ground as was done in the present study. The procedure of taking two cores from opposite sides of each tree for each test fungus worked well. If five or six fungi were employed, a single core from each tree for each fungus would be good sampling, although it would fail to provide a base for testing the significance of differences among individual trees. The use of several fungi is advisable, and for any broadleaved tree species at least one white rot fungus such as *Polyporus versicolor* should be included among the test fungi. Of the brown rot fungi *Poria monticola* is excellent for general use.

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CHEMICAL REMOVAL OF ENCRUSTANTS FROM DEW-RETTED HEMP FIBER [1]

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INTRODUCTION

The bast fibers of dew-retted hemp (*Cannabis sativa* L.) are at a disadvantage for textile use because of the presence of encrusting materials. These encrustants, which make up roughly 30 percent of the retted hemp fiber, consist largely of lignin, pentosans, pectic substances, protein, and ash (*5*).[3] They serve as a binding material which holds the fiber bundles together and add strength, but they are rather loosely held and in time and with handling of the fiber they partially slough off. As a result of the great variation in these encrusting substances, physical testing of bast fibers is very difficult. Therefore it is believed that a more uniform basis which would permit a better comparison of fibers should be found. Most chemical degumming treatments are partly effective for removing the encrustants, but some of them cause rather great degradation of the primary constituent of the fiber, cellulose. Reagents which degrade the cellulose chain would, of course, be of little value for this kind of treatment.

Alcohols have been used in pulping wood, which is essentially getting down to the cellulose. Aronovsky and Gortner (*1*) found that alcohols with at least four carbon atoms of which at least three are in a straight chain are best suited for pulping. Organic alcohols if effective in removal of encrustants because of their solvent action should also cause the least cellulose degradation and are worthy of a thorough trial as degumming agents.

In order to ascertain what chemical treatments would remove encrustants from dew-retted hemp fiber and their effects on the fiber, the tests reported herein were made.

MATERIAL AND METHODS

Dew-retted hemp fiber considered to be of about average length was used in the tests. It was air-dried and cut in about 2-inch lengths before treatment with a degumming reagent. Each sample was

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refluxed for an experimental period in a large test tube with a cold finger for condensing vapors. After refluxing was complete, the degumming reagent was washed off, usually with water only; if the reagent was practically insoluble in water, however, a preliminary washing was given with ethanol to facilitate the removal. The fibers were then pressed between paper towels and dried.

Part of most samples were subsequently treated by boiling 1 hour in 1-percent sodium hydroxide and then ½ hour in 4-percent boric acid. The boric acid was used to free the fiber of sodium hydroxide, which interfered with the nitration and determination of the degree of polymerization. Since the latter concerns the cellulose chain, most of the determinations were made on samples from which the encrustants had been removed.

The nitration method used was essentially that of Berl (*2*) except that the nitration was carried out at 10° C. rather than at room temperature. Cellulose nitrate was dissolved in butyl acetate to make a 0.25-percent solution. This was accomplished by revolving between two parallel bars a small glass jar containing the mixture and steel bearings. After solution the cellulose nitrate was filtered through a coarse-fritted glass crucible. The viscometer used was that of Cannon and Fenske (*3*), the viscosity being determined at 25°±0.02° with the time of flow 100 seconds or more.

Calculations from degree of polymerization were based on the following formula of Coppick (*4*):

$$[\eta] = \frac{\ln \eta r}{c} \ (1+0.5c)$$

where

$[\eta]$ = intrinsic viscosity
$\ln$ = natural log
ηr = relative viscosity
c = concentration in grams per 100 ml.

The degree of polymerization was calculated from Kraemer's formula (*6*):

$$\text{Degree of polymerization} = K[\eta]$$

where

$$K = 270.$$

Degree of polymerization, which is affected by various factors, indicates how well fibers withstand treatment to remove encrustants. A low value is evidence of cellulose degradation and therefore of decrease in fiber strength.

Nitrogen in the cellulose nitrate was determined by the Lunge nitrometer.

The percentage nitrogen in the fiber after nitration, which shows the degree of nitration, is dependent on how well the degumming and alkali treatments remove the encrustant from the primary constituent, cellulose.

Breaking strength of the fiber was measured on the Scott tester, model D. H. After loose ends were removed, the sample was cut in 25-cm. lengths and conditioned at 65±1 percent relative humidity. Spool-type clamps, measuring 7.5 cm. from center to center, were

used. Ten breaking tests were made on each sample, and the average breaking strength was reported in kilograms per gram of fiber.

RESULTS

The role played by encrustants in the physical strength of raw commercial bast fibers was determined by testing duplicate samples of dew-retted hemp fiber with the encrustants intact and with the encrustants removed by boiling 1 hour in 1-percent sodium hydroxide and then ½ hour in 4-percent boric acid. The fiber with encrustants
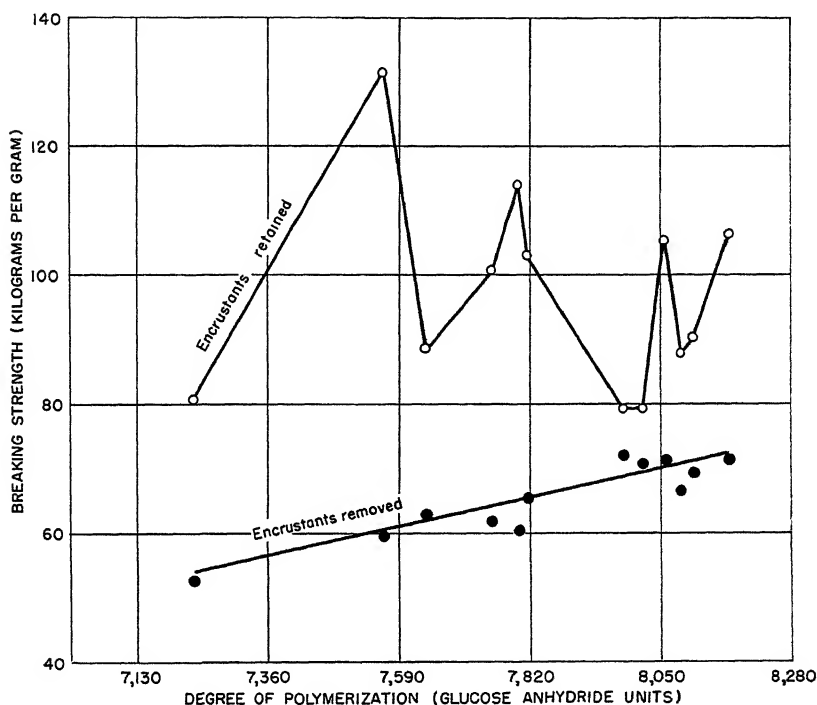
FIGURE 1.—Relation between degree of polymerization and breaking strength of dew-retted hemp fibers with and without encrustants.

removed had a much lower breaking strength than that with the encrustants present (fig. 1). Cellulose in the samples with encrustants averaged 70.3 percent and in those without encrustants 95.7 percent. Breaking strength and degree of polymerization were very closely correlated in the samples without encrustants ($r=0.94$).

The data presented in figure 1 help to answer several questions about the retting process. The over-all variability in strength of fibers with encrustants removed, as measured by degree of polymerization, was relatively slight, a range of approximately 10 percent. The strength of such fibers, like that of most commercial fibers, is essentially that of the primary constituent, cellulose, and depends on length of cellulose chain. Such strength, measured by degree of polymerization, is average strength, which is not affected by weak spots to the

same degree as strength measured by mechanical breaking. The data for fibers with encrustants present substantiate the already known fact that dew retting is irregular and indicate that much of the non-uniformity in quality and strength of such fiber is due to irregularity in encrustant removal.

The results of treating dew-retted hemp fibers with various degumming reagents for different periods (table 1) indicate that they vary in effectiveness and that care in selecting is essential.

TABLE 1.—*Effect of chemical removal of encrustants from dew-retted hemp fiber on degree of polymerization and extent of nitration*

[Alkali boil = boiling 1 hour in 1-percent sodium hydroxide and then ½ hour in 4-percent boric acid]

Sample No.	Degumming reagent			Degree of polymerization in glucose anhydride units		Nitrogen	
	Description [1]	Time of boiling	After degumming treatment	After degumming treatment plus alkali boil	After degumming treatment	After degumming treatment plus alkali boil
		Hours	*Number*	*Number*	*Percent*	*Percent*
1	None		[2] 6,722	[3] 7,790	12.30	12 92
2	Sodium chlorite (1 percent in 3-percent acetic acid at 70° C.)	½	7,767		13.10	
	Sodium carbonate: [4]					
3	1 percent	1	8,117		12.78	
4	3 percent	1	8,110		12.67	
5	5 percent	1	8,119		12.73	
6	10 percent	1	8,326		12.62	
	Sodium sulfite: [4]					
7	1 percent	1	6,813		12 53	
8	3 percent	1	6,548		12.70	
	Sodium borate: [4]					
9	1 percent	1	6,504		12.15	
10	3 percent	1	7,033		12.59	
11	Sodium hydroxide (2 percent in 95-percent ethanol)	1	7,452		12.95	
	Monoethanolamine·					
12	3 percent in 95-percent ethanol	1	7,026	7,158	12.60	13.05
13	2.5 percent in glycerol	1	7,222	7,280	13.05	13.10
14	5 percent in glycerol	1	6,831	6,033	13 04	13.04
15	do	2	6,491	6,258	12.92	13.09
16	Xylene	1	7,383	7,335	12.50	13.15
17	Methanol	1	6,718	7,721	12.56	13.01
18	Ethanol (95 percent)	1	6,722	7,659	12.40	13.17
19	Butanol	1	7,473	7,944	12.56	13.22
20	Isobutanol	1	7,729	7,590	12.53	13.07
21	Amyl alcohol	1	7,061	7,526	12.10	12.80
22	Methanol and butanol (equal parts)	1	7,606	8,096	12.38	12.96
23	Ethanol and butanol (equal parts)	1	7,510	8,211	12.44	13.12
24	Butanol and glycerol (equal parts)	1	7,326	8,211	12 10	13.30
25	Ethanol, butanol, and glycerol (equal parts)	1	6,941	7,832	12.22	13.24
26	Ethylene glycol	1	6,918	6,941	12.75	12.85
27	do	4	7,107	7,015	12.98	12.98
28	Ethylene glycol followed by bleach [5]	4	6,481	6,194	13.07	13.17
29	Ethylene glycol	8	7,107	6,364	12.85	12.80
30	Ethylene glycol followed by bleach [5]	8	6,100	6,318	13 29	13.23
31	Glycerol	1	7,107	7,121	12.48	12.70
32	do	4	5,994	6,539	12 80	12.85
33	Glycerol followed by bleach [5]	4	6,472	5,842	13.12	13.24
34	Glycerol	8	6,334	6,550	12.83	13.06
35	Glycerol followed by bleach [5]	8	5,948	5,771	13.04	13.04

[1] Reagent 100 percent unless indicated otherwise.
[2] Value for original fiber.
[3] Value after alkali boil only.
[4] Treatment with reagent followed by ½-hour boiling in boric acid.
[5] Bleach = ½ hour in sodium chlorite in 3-percent acetic acid at 70° C.

The first 11 samples show the effects of using inorganic alkalies and salts to remove the encrusting materials. Sodium hydroxide was the most effective of the inorganic compounds, excluding the sodium chlorite bleach, in the removal of encrustants as measured by the percentage of nitrogen. Sample 1 before the alkali boil had a lower degree of polymerization because of two factors: (1) greater nitration of pentosans, which would have a tendency to lower the degree of polymerization, and (2) increase of polymerization with increased nitration. Also some degumming reagents may more selectively remove the lower fraction and thus favor a higher degree of polymerization in the treated fiber. Bleaching with sodium chlorite (sample 2) increased nitration and gave a high degree of polymerization, which indicated little break-down of the cellulose chain. Samples 3 to 6, treated with different concentrations of sodium carbonate, showed less removal of encrustants and a higher degree of polymerization, indicating that there was some degradation of the sample treated with sodium hydroxide (sample 1). The concentration of sodium carbonate had little effect on encrustants or the cellulose chain, no doubt because of the high buffer action of this reagent. Treatment with sodium sulfite resulted in increased removal of encrustants as measured by nitration and also greater degradation of cellulose as the concentration of the reagent increased. Furthermore, the sulfite-treated samples showed more cellulose break-down than those treated with sodium hydroxide or sodium carbonate. Sodium borate acted similarly to sodium sulfite, although it was not so effective in the removal of encrustants. The higher degree of polymerization at the 3-percent level was perhaps due to increased nitration. Sodium hydroxide in ethanol resulted in good removal of encrustants and little degradation of cellulose.

The action of monoethanolamine as a delignifier of wood tissue (7) has been known for some time, and the same chemical has been used in the determination of cellulose (8). Samples 12 through 15 show the effects of using monoethanolamine in ethanol or glycerol. The amine in glycerol has definite advantages over that in ethanol. Monoethanolamine in glycerol was used above its boiling point, and it was therefore more effective. The 2.5-percent concentration did just as well as the 5-percent one in removing the encrustants, and 2 hours had no advantage over 1 hour. Monoethanolamine caused some degradation of cellulose, although not so much as sodium sulfite and sodium borate. Judged by the percentage of nitrogen, the additional removal of encrustants with sodium hydroxide did not cause additional nitration; however, there was greater cellulose degradation in samples 14 and 15 after alkali boil. This greater break-down of cellulose in the higher concentration of monoethanolamine and after the longer boiling time is evidence of better opening up of the fiber bundle for the action of sodium hydroxide.

Samples 16 through 25 show the effects of treatment with xylene or certain alcohols singly and in combination on dew-retted hemp fiber. While xylene is not an alcohol, it acted very much like the alcohols in that it opened up the fibers for more effective action of other reagents such as sodium hydroxide. The degree of polymerization was generally higher when the alcohol was followed by sodium hydroxide. A possible explanation is that there was less extraneous material

connected with the fiber and therefore less low-viscosity material. The higher nitrogen values are evidence of purer cellulose, or better removal of encrustants. A lower nitrogen value for the alcohols used in combination than for those used singly indicates lower removal of encrustants, or a suppressing action by the combination. The combinations of alcohol gave very high degrees of polymerization when followed by the sodium hydroxide boil. A possible explanation may be the better removal of the shorter chained molecules such as pentosan. There has been evidence that some of the pentosan fractions are held much more tenaciously than others by cellulose. The higher alcohols may expose this hard-to-remove fraction to easier removal by other reagents. Cellulose in fiber boiled in four of the alcohols singly—ethanol, butanol, ethylene glycol, and glycerol—averaged 91.5 percent, while in these same samples after boiling for 1 hour in 1-percent sodium hydroxide it averaged 97.4 percent.

Ethylene glycol and glycerol were the two highest boiling alcohols used for encrustant removal. The effects of these two alcohols alone with boiling times of 1 to 8 hours or followed by the sodium hydroxide boil, bleach, or a combination of the two are shown by samples 26 through 35. Ethylene glycol was the most effective of the alcohols in the removal of encrustants during a 1-hour boiling period; however, the lower degree of polymerization indicates greater degradation of cellulose. Glycerol also resulted in cellulose destruction as measured by degree of polymerization. The degradation of the cellulose chain by treatment with glycerol seems to be due to the greater heat rather than to the chemical action, since high temperatures are known to degrade cellulose. Ethylene glycol alone caused no greater degradation in 8 hours than in 1 hour. These two high-boiling alcohols may have some dehydrating action which makes the fibers more subject to oxidation and in general to greater break-down when the alcohol treatment is followed by the sodium hydroxide boil, a bleach, or a combination of the two.

SUMMARY AND CONCLUSIONS

In order to ascertain the effect of degumming dew retted hemp fibers on cellulose degradation, more than 30 treatments were tested with and without subsequent boiling with sodium hydroxide.

The tests showed that the formation of cellulose nitrate and the subsequent determination of degree of polymerization and percentage of nitrogen may be used as an index to encrustant removal and resulting fiber degradation and quality. Sodium hydroxide and sodium carbonate are good chemicals to use in removing encrustants. The former is more effective, but it causes some degradation of the cellulose chain. Increasing the concentration of sodium carbonate over the 1-percent level did not give better removal of encrustants or cause greater break-down of cellulose. A 2.5-percent concentration of monoethanolamine in glycerol gave excellent removal of encrustants, although it caused some cellulose degradation. The lower boiling alcohols such as methanol, ethanol, butanol, isobutanol, and amyl alcohol are not very effective in removing encrustants; however, they do open up the fiber and allow other mild reagents to act more effectively. Of the two high-boiling alcohols (ethylene glycol and glycerol), ethylene glycol was better in removal of encrustants and caused

slightly less degradation of cellulose. The higher boiling alcohols have a tendency to make the fiber more subject to oxidation in bleaching. Increasing the boiling time over 1 hour did not greatly increase the removal of encrustants, but, especially in case of glycerol, it did cause a lowering of the degree of polymerization.

These tests indicate that degumming of bast fibers can be undertaken with a minimum of cellulose degradation and that encrustant removal to a common cellulose base will result in more uniform physical testing.

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THE EFFECT OF AIR TEMPERATURE ON VIRUS CONCENTRATION AND LEAF MORPHOLOGY OF MOSAIC-INFECTED HORSERADISH [1]

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INTRODUCTION

Mosaic of horseradish (*Armoracia rusticana* Gaertn., Mey and Scherb.) is incited by one or more strains of turnip virus 1.[3][4] In a recent paper[4] it was shown that in plants naturally infected with mosaic, symptoms tended to become masked completely at 28° C., they were progressively more pronounced at 24° and 20°, and were severe and persistent at 16°. This was contrary to the behavior of other strains of the same virus which incited mosaic on cabbage. Symptoms caused by these were most severe at 28°, were progressively less pronounced at 24° and 20°, and tended to become masked at 16°.[5] In cabbage the concentration of strains of turnip virus 1 was correlated with the severity of symptoms, the greatest concentration occurring with the most severe symptoms.[6] It became of interest, therefore, to determine the effect of air temperature on the concentration of the horseradish virus in horseradish. In the course of the experiments it was observed that temperature had a marked effect on leaf morphology, an effect apparently independent of that on virus concentration.

MATERIALS AND METHODS

Horseradish roots were obtained from the planting stock of a commercial grower of East St. Louis, Ill. They were planted out in February 1947, in greenhouse compost in 12-inch clay pots, seven plants per pot. After 3 weeks' growth at 22° C. they were placed at constant air temperatures of 16°, 20°, 24°, and 28° for 30 days, when

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an assay was made for virus concentration in plants at 16° and 28°. During the summer of 1947 the plants were transplanted to the field. In October they were lifted and roots from each of the four groups were replanted to pots and placed at each of the four greenhouse temperatures mentioned above.

In preparing inoculum for an assay of virus concentration, a composite sample of 500 milligrams of tissue was selected from the youngest leaves of each pot of a given treatment. Only marginal leaf tissue from closely comparable leaves was used, care being exercised to avoid midrib tissue. The tissue was macerated in 10 cubic centimeters of distilled water until it was macroscopically homogeneous. The assay consisted of half-leaf inoculations to tobacco (*Nicotiana tabacum* L. var. Connecticut Havana No. 38) on which the virus produces only necrotic local lesions. Tobacco plants were evenly sprinkled with carborundum before the inoculum was applied with a glass spatula.

When only one comparison was to be made, two to five tobacco plants with three leaves each were used. When all possible comparisons of the four temperatures were desired, seven tobacco plants of three leaves each were used in an incomplete block design.[7][8] The following arrangement was used in which horizontal rows represent tobacco plants and vertical columns represent leaf positions on these plants.

A   B   C   D   E   F   G
B   D   F   E   G   A   C
C   F   E   A   B   G   D

Since the four temperatures allowed only six different comparisons, one treatment was duplicated each time. It can be seen that each treatment occurred once at each leaf position and once on the same plant with each of the other treatments. Occasional uniformity

TABLE 1.—*Results of 3 uniformity trials to test the half-leaf carborundum method used in assaying virus concentration*

Hypothetical half-leaf comparisons		Average number of local lesions in 3 replicates in trial indicated		
		Trial 1 [1]	Trial 2 [1]	Trial 3 [1]
A	Left	59.3	83.0	84 3
	Right	84.7	199.7	75.0
B	Left	39.3	141.3	64.7
	Right	42.0	178.7	62.3
C	Left	39 0	149 0	63.0
	Right	45.3	153.0	64.0
D	Left	41.0	192 3	125.7
	Right	54.7	214.7	139.0
E	Left	39.7	163.0	109.3
	Right	38.7	179.7	108.0
F	Left	47.0	139.3	93 0
	Right	59.7	151.0	83.7
G	Left	49.0	97.0	54.3
	Right	52.3	87.7	67.0

[1] No significant difference between treatments occurred in any of the trials.

[7] YOUDEN, W. J. USE OF INCOMPLETE BLOCK REPLICATIONS IN ESTIMATING TOBACCO-MOSAIC VIRUS. Boyce Thompson Inst. Contrib. 9: 41–48, illus. 1937.
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trials in which the same experimental design was used were run to assure the reliability of the half-leaf carborundum method. In these tests the same inoculum was applied to both halves of all leaves. Results of three such trials are given in table 1. It can be seen that in each of the three trials the half-leaf method was very reliable.

EXPERIMENTAL RESULTS

EFFECT OF TEMPERATURE ON SYMPTOMS AND LEAF MORPHOLOGY

When horseradish roots were forced in the greenhouse, symptoms appeared first at 28° and last at 16°. Initial symptoms consisted of a blotchy chlorotic mottle which sometimes was preceded by chlorotic vein clearing. Gradually, the symptoms at 28° and 24° became less intense and ultimately were completely masked. Leaves became dark green and showed no mottling and only rare necrotic streaking. At the same time symptoms at 20° and 16° became more intense, the blotchy mottle being replaced by a severe diffuse, yellow mottle and conspicuous necrotic ringing and streaking. When tops were removed and temperatures reversed, a corresponding complete reversal in symptom intensity ultimately occurred. When plants were taken from 28° and 16° and grown at the same temperature, they gradually became indistinguishable.

Field observations have shown a fernlike leaf to be very prevalent in horseradish. In the present studies this pinnatifid leaf type was found to be very prevalent at 16° C. but practically nonexistent at 28° (fig. 1). A perfect gradient existed from 28° to 16°, going from broadly laminate to pinnatifid leaves, respectively. When tops were removed and temperatures reversed, leaf type for the first few days was characteristic of the temperature at which the plants had grown before the temperature reversal (fig. 2). This was no doubt due to the fact that the leaf primordia for these early leaves were already initiated at the time of the temperature reversal. Sixty days after the temperature change, however, a complete reversal in leaf type had occurred, pinnatifid leaves being formed at 16° and laminate leaves at 28°. When plants from each temperature showing the characteristic leaf type were planted together in the field, they gradually became indistinguishable. When each group was returned to the different temperatures in the greenhouses, the same response to temperature occurred.

To ascertain if this fernlike leaf was correlated with virus concentration the following tests were made. Plants were grown at 28° and 16° C. until the respective leaf types were well established. The groups were divided into two parts each, the tops were removed, and temperatures were reversed on one-half of each group. Two weeks later both leaf types at both temperatures were present. Assays of virus concentrations were made between the two-leaf types at both 28° and 16°. No significant difference was found in the virus concentration between the two-leaf types at either temperature. However, fernlike leaves at 16° had a higher virus titer than laminate leaves at 28° but fernlike leaves at 28° had a lower virus titer than laminate leaves at 16°. Thus it appears that the temperature effect on leaf morphology is independent of that on virus concentration detailed in the following section.

FIGURE 1.—Types of leaves produced on mosaic-infected horseradish at 28° C (*A*) and 16° C. (*B*).

EFFECT OF TEMPERATURE ON VIRUS CONCENTRATION IN HORSERADISH

Thirty days after the plants were placed at the different temperature levels in the spring of 1947, those at 16° and 28° C. were assayed for virus concentration. The following results were obtained.

	Average number of local lesions in half-leaf comparisons in 16 replications
16° C	37. 9
28° C	4. 9
Difference required for significance (19:1)	15. 7
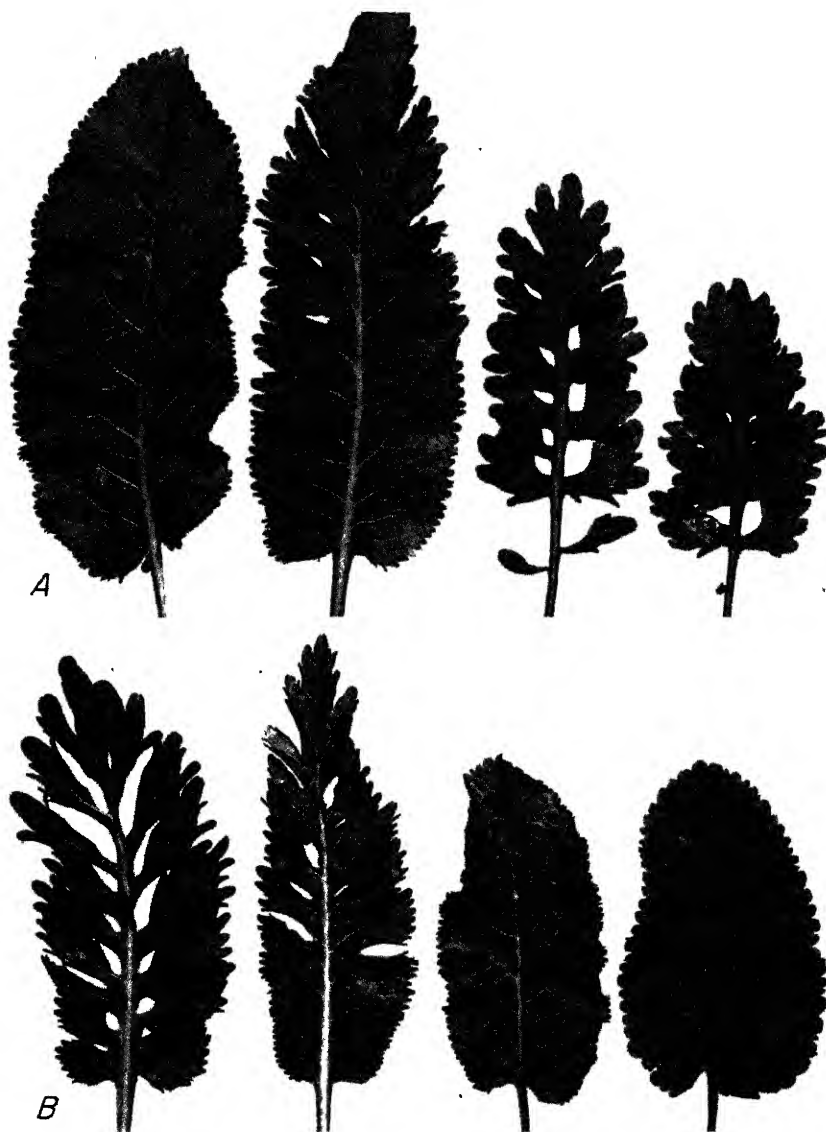
FIGURE 2.—Representative leaf types of mosaic-infected horseradish from plants grown at, left to right, 28°, 24°, 20°, 16°, respectively: *A*, from plants grown for 60 days at their respective temperatures; *B*, from plants grown for only 2 weeks at temperatures shown and prior to that for 60 days at, left to right, 16°, 20°, 24°, 28°, respectively.

It can be seen that in this single assay the virus concentration appeared to be much higher in plants grown at 16° than in those grown at 28°.

In October 1947, the plants were returned from the field and roots from each group were placed at the four different temperatures.

TABLE 2.—*Comparative concentration of horseradish mosaic virus in horseradish plants after growing for 60 days at 16°, 20°, 24°, 28° C., December 1947*

Half-leaf temperature comparisons (° C.)	Average number of local lesions in 3 replicates from inoculum of group [1]—				Total of 4 groups	F value for half-leaf comparisons [2]
	1	2	3	4		
28	2.7	2.3	1 0	0 7	6.7	12 2**
24	5 7	9 3	12 7	4 3	32 0	
28	5 0	3.7	2 7	2.3	13.7	24.1**
20	43 3	16 0	13 3	24 7	97 3	
28	4.0	1.3	1.7	1.0	8 0	80 2**
16	251.7	179 0	207.7	112 7	775 1	
24	4.3	3 0	10 7	4.3	22 3	8 7*
20	62 3	14 7	9 7	21 3	108.0	
24	9 3	5 7	11 7	5 0	31 7	72. 6**
16	205.0	200 7	212 7	128 3	746 7	
20	78.3	12.3	11.3	5.7	107.6	28 7**
16	148.3	165.0	231.3	132 7	677.3	

[1] Groups 1, 2, 3, 4 correspond, respectively, to lots grown at 16°, 20°, 24°, and 28° C. during the spring of 1947 and in the field during the summer of 1947.
[2] *values are significant at the 5-percent level. **values are significant at the 1-percent level.

After 60 days, assays were made from all groups at all temperatures, each group being considered separately and being tested on seven tobacco plants according to the design given on p. 162. The results are presented in table 2. A study of the data reveals that a gradient of virus concentration existed between the temperatures, with extremely low concentrations at 28° and high concentrations at 16°. The greatest break occurred between 20° and 16°. The four trials were very consistent in their trend with no reversal to be accounted for. The group grown at 28° in the spring of 1947 (group 4) gave fewer lesions than the others but it is doubted if that was a carry-over effect of the high temperature. In another experiment the virus titer in young leaves at 16° was found to be greater than that in old leaves at 28°.

Immediately after the above assays were made, all top growth was removed from the plants and the pots from 16°, 20°, 24°, and 28° houses were transferred to houses of 28°, 24°, 20°, and 16°, respectively. Assays were made from the plants at 28° and 16° of groups 1, 2, 3, and 4 on the fifteenth, eighteenth, twenty-seventh, and twenty-ninth days, respectively, after reversal of temperatures. The results are given in table 3. It can be seen that even after only 15 days at the new temperatures the virus concentration of 16° was significantly higher than that at 28°. In all four groups this was the case.

TABLE 3.—*Comparative concentrations of horesradish mosaic virus in horseradish plants grown for 60 days at 16° and 28° C., and then for 2 to 4 weeks at 28° and 16°, respectively*

Temperature after reversal (°C.)	Average number of local lesions in 6 replicates from inoculum from group [1]—			
	1	2	3	4
16	73.0	89.0	173.5	109.3
28	34.8	19 2	5 5	32.1
L. S. D. (19:1)	16.5	30 1	32 6	28.9

[1] Assay from group 1 made 15 days after temperature reversal. Assay from group 2 made 18 days after temperature reversal. Assay from group 3 made 27 days after temperature reversal. Assay from group 4 made 29 days after temperature reversal.

Sixty days after the temperature reversal a further assay of virus concentration was made from plants growing at the 16° and 28° temperatures. In this assay, inoculum was prepared from the very youngest leaves of each group and also from the oldest leaves. The oldest leaves would have been formed soon after the temperature reversal. Because of a shortage of suitable assay plants groups 1 and 2 were combined and groups 3 and 4 were combined. The results, which are given in table 4, show that the virus was still in significantly higher concentration at 16° than at 28° but that the concentration in young leaves grown at 16° was considerably lower than in previous tests. In one case, the concentration in young leaves at 16° was significantly greater than that at 28° only at the 5-percent level of probability. No explanation can be given for this drop in concentration other than that the plants by that time were not in a very active state of growth. It is also notable that the virus concentrations from the combination of groups 3 and 4 were higher than from groups 1 and 2, but some of this difference could be accounted for in the difference between assay plants used.

TABLE 4.—*Comparative concentrations of horseradish mosaic virus in horseradish plants grown for 60 days at 16° and 28° C. and then for 60 days at 28° and 16°, respectively*

| Temperature after reversal (° C.) | Average number of local lesions in 6 replicates for group and inoculum indicated | | | |
| | Groups 1 and 2 combined [1] | | Groups 3 and 4 combined [1] | |
	Inoculum from youngest leaves	Inoculum from oldest leaves	Inoculum from youngest leaves	Inoculum from oldest leaves
16	14 7	93.1	26.1	356.0
28	6 3	5.1	7.8	101.9
L. S. D. (19.1)	4.8	43 6	16.2	57.8

[1] For explanation of groupings see footnote of table 3.

It was thought that perhaps the lower concentration at 28° than at 16° might be due to the presence of an inhibitor in expressed sap from plants grown at 28°. To check this point an experiment was run in which inoculum from each temperature was tested in a series of dilutions with the idea that if an inhibitor were present one might neutralize its activity by dilutions low enough to leave the virus active. The results are given in table 5. It can be seen that this test gave no indication of the presence of an inhibitor.

As a further test for the presence of an inhibitor inoculum from plants grown at 28° was divided into two equal portions. One portion was treated with 10 percent Nuchar W adsorbent; the other was untreated. When the two inocula were compared in half-leaf inoculations, there was no significant difference in the number of local lesions produced. Thus, if an inhibitor was present it was not indicated by this test.

TABLE 5.—*Results of dilution of inoculum taken from plants grown at 28° and 16° C.*

[Dilutions compared in each case in half-leaf inoculations with undiluted control]

| Extent of dilution | Average number of local lesions in 9 replicates | | | |
| | Inoculum from 28° | | Inoculum from 16° | |
	Control	Diluted	Control	Diluted
1-10	18.3	2.9	165.3	88 8
1-100	17 9	0	195 0	3.9
1-1,000	17.7	0	188.7	2.1

EFFECT OF TEMPERATURE ON THE CONCENTRATION OF THE HORSERADISH VIRUS IN RAPE

Horseradish viruses produce severe symptoms on plants of Dwarf Essex rape (*Brassica napus* L.).[9] To ascertain whether the virus in question showed a differential temperature response in rape the following experiment was performed. Forty plants of rape in the first true-leaf stage were inoculated with a composite inoculum from all horseradish plants under study. After 5 days' incubation at 22°, they were divided equally between the 16° and 28° houses. After 35 days, assays were made, comparing inocula from 16° and 28° plants both from youngest leaves (tenth leaf above cotyledons at 16°; twelfth leaf above cotyledons at 28°) and from old leaves (fifth leaf above cotyledons at both temperatures). The results, which are given in table 6, show that in young leaves the virus was in higher concentration at 28° than at 16° but in old leaves the reverse was true. In additional inoculations not shown in [table 6 it was found that at both temperatures the concentration was greater in old leaves than in young leaves although significant only at the 5-percent level for the 28° plants. A much greater difference occurred between the young and old leaves at 16°.

TABLE 6.—*Comparative concentrations of horseradish virus in rape plants grown at 16° and 28° for 35 days prior to assay*

| Temperature (°C.) | Average number of local lesions in 12 replicates from inoculum from— | |
	Young leaves	Old leaves
16	41.8	234.3
28	75.5	112.2
L. S. D. (19.1)	12.2	35.0

EFFORTS TO INACTIVATE THE VIRUS IN HORSERADISH ROOTS AND PLANTS BY TEMPERATURE TREATMENTS

Since the thermal inactivation point of the horseradish virus was found to be slightly under 60° C. for 10 minutes, efforts were made to inactivate it in live roots by hot-water treatments and in growing plants by exposure to excessively high-air temperatures. Both experiments were unsuccessful, as shown by the data in table 7.

[9] See footnote 4, p. 161.

Top growth was burned badly by the high-air temperatures but terminal growing points were not killed and subsequent growth developed conspicuous symptoms. The first growth produced after the treated roots were planted also showed conspicuous symptoms.

TABLE 7.—*Results of hot water treatments of horseradish planting roots and hot air treatments of growing horseradish plants*

Time and temperature (°C) of treatment	Number of roots or plants treated	Number of survivors	Number of survivors showing symptoms	Virus recovery test from survivors
Hot water:				
Untreated_____	20	20	20	Positive.
10 minutes at 65°_____	4	0	_____	
10 minutes at 60°_____	10	0	_____	
10 minutes at 55°_____	10	0	_____	
20 minutes at 50°_____	[1] 8	4	4	Do.
30 minutes at 50°_____	10	0	_____	
30 minutes at 45°_____	8	8	8	Do.
·120 minutes at 45°_____	10	3	3	Do.
45 minutes at 40°_____	4	4	4	Do.
240 minutes at 40°_____	10	2	2	Do.
Hot air:				
48 hours at 50°_____	7	7	7	Do.
72 hours at 50°_____	7	7	7	Do.
96 hours at 50°_____	7	7	7	Do.
120 hours at 50°_____	7	7	7	Do.

[1] Roots of three-fourths inch diameter were used for this test. In all other tests roots of one-half inch diameter were used.

DISCUSSION

The data presented herein are interpreted as indicating different levels of virus concentration in horseradish plants growing at different temperatures. However, the possibility cannot be overlooked that a virus inhibitor may be present in horseradish and may occur more at one temperature than at another. Although two tests failed to indicate any inhibitor the possibility has not been fully explored.

The development of more conspicuous and more severe symptoms of horseradish mosaic in the field in late fall is explainable by results reported in this paper. At high air temperatures symptoms are masked and the concentration of the virus is relatively low. At low temperatures symptoms are persistent and virus concentration is relatively high. The author has never observed horseradish growing in areas where average air temperatures would reach or surpass 28° C. (e. g., the East St. Louis area), but in Wisconsin and western Washington it has been observed that symptoms are most marked late in the fall. It would not be surprising if infected horseradish growing in southern areas would appear perfectly healthy in mid-summer but quite diseased in late fall. This would be the case, however, only if other strains of horseradish mosaic virus showed the same temperature response as the one studied in this report.

The correlation of symptom severity with virus concentration in horseradish is similar to that reported for certain cabbage strains of turnip virus 1[10] except that symptoms on cabbage were more pronounced and viruses were in higher concentrations at high temperatures than at low temperatures. The concentration of the cabbage

[10] See footnote 6, p. 161.

strains was not determined in any other host nor was that of the horseradish virus determined in cabbage. The different temperature responses shown by the horseradish strain and the cabbage strains indicate that all strains of turnip virus 1 are not favored by high air temperatures on all hosts as suggested by Pound and Walker.[11]

The fact that the horseradish virus occurred in higher concentration at 28° than at 16° in young leaves of rape but that the reverse occurred in old leaves further indicates that the effect of temperature depends upon the specific host-virus complex and may be due to the influence of the environal factor on the host, the virus, or both. It also indicates that in studies of physical properties where virus concentration is a limiting factor, one should give prime consideration to age of tissue involved as well as temperature at which the plants were grown.

No explanation is offered for the factors involved in the temperature effect on leaf morphology. Data presented herein seem to indicate that the reaction is independent of virus activity. This is further borne out by the fact that a horseradish clone from which no virus has been isolated and which has not become infected upon inoculation also develops the fernlike leaves. It is possible that temperature affects the physiology of the plant relative to some growth regulating substance.

SUMMARY

A virus causing horseradish mosaic was found to occur in much higher concentration in horseradish grown in greenhouses at 16° C. than at 28°. This was also true for old leaves of rape plants infected with the horseradish virus, but for young leaves of rape the concentration was higher at 28°.

When horseradish plants growing at different temperatures and showing a gradient of virus concentration were reversed in position, the plants being moved from 16° to 28°, etc., a corresponding reversal in virus concentration occurred.

A marked temperature effect on leaf morphology of horseradish was also recorded. At high temperatures, the prevailing leaf type was broadly laminate. At low temperatures leaves were exceedingly pinnatifid, often being reduced to extreme fernleaf structures. At intermediate temperatures intermediate types occurred. A reversal of temperatures resulted in a corresponding reversal in leaf type. The temperature effect on leaf type is thought to be independent of that on virus concentration.

Efforts to free horseradish roots and plants from the virus by heat treatments in hot water and hot air, respectively, were unsuccessful.

[11] See footnote 5, p. 161.

BREEDING LOW-NICOTINE TOBACCO [1]

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INTRODUCTION

In 1933 the Kentucky Agricultural Experiment Station received from J. B. Hutson, of the United States Department of Agriculture, then stationed in Germany, seven lots of Havana and Cuba cigar tobacco varieties low to very low in nicotine content. These seeds had been obtained from Dr. Erwin Baur, director of the Kaiser Wilhelm Institute. So far as the writer was aware, there were at that time no varieties of cigarette or pipe tobacco of low nicotine content. It was decided, therefore, to develop a variety or varieties of cigarette tobacco of low nicotine content to meet a possible demand for such a tobacco as a health measure. It was further decided that this variety should carry the recessive color factors of burley because burley is a light-colored tobacco when cured and light color is commonly associated in the mind of the public with mildness.

MATERIALS AND METHODS

Three strains of Cuba, designated 30/33, 31/33, and 32/33, each listed as very low in nicotine, were used as the source of the low-nicotine character. In 1934, analyses of these varieties showed that they contained 0.19, 0.05, and 0.11 percent nicotine, and in 1935, 0.18, 0.22, and 0.21 percent, respectively. These varieties were crossed with Ky 5, Ky 7, Ky 14, and Ky 16—all black-root-rot-resistant varieties of burley tobacco. In 1934 these varieties analyzed 2.18, 2.10, 1.37, and 2.18 percent nicotine, respectively.

All nicotine determinations were made on leaves from the middle of field-grown plants as these leaves are usually the highest in nicotine in tobacco harvested and cured like burley. During the past several years the tobacco has been grown on more fertile soil than in the early years, and the nicotine content of Ky 16, used as a control, has been higher. From 1934 to 1942 the nicotine determinations were made by members of the Department of Chemistry of the experiment station, who used the method of the Association of Official Agricultural Chemists (1, *p.* 64).[2] In 1943 and 1944 Markwood's (*10*) green disk method was used and determinations were made by E. M. Johnson. Since then the determinations have been made under the

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[1] Received for publication May 17, 1948.
[2] Italic numbers in parentheses refer to Literature Cited, p. 180.

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direction of R. B. Griffith who has used a rapid modification of the A. O. A. C. method (*6*). In 1947 determinations were made by the method of Bowen and Barthel (*3*).

EXPERIMENTAL RESULTS

CROSSES

The F_1 generation of the dark cigar tobacco by the light-colored burley was all dark, as would be expected. A single determination of nicotine in an F_1 30/33 × Ky 7 showed that it had 3.18 percent when Ky 16 had 1.73 percent and the low-nicotine parent had 0.22 percent. This suggests that the high-nicotine factor is completely dominant over low nicotine. The difference in nicotine content between the F_1 hybrid and Ky 16 is not significant.

The F_2 generation segregated with respect to plant color, producing approximately 15 dark plants to 1 light-colored plant. There was also segregation with respect to nicotine content, with a range from plants with no nicotine to 2.82 percent in a total of 345 burley-colored plants tested (table 1).

TABLE 1.—*Nicotine content of burley-colored F_2 plants from low-nicotine cigar tobaccos crossed with burley, 1935*

Nicotine (percent)	31/33×Ky 5	30/33×Ky 7	31/33×Ky 14	30/33×Ky 16	32/33×Ky 14	Total
0	2	3	4	2	14	25
.1	14	11	20	10	11	66
.5	9	20	23	26	21	99
1 0	22	17	22	18	12	91
1.5	11	15	4	6	3	39
2.0	10	8	5	2	0	25
Total plants	68	74	78	64	61	345

There appears to be no sharp segregation into low-nicotine and high-nicotine content plants as claimed by Hackbarth and Sengbusch (*9*), who concluded that the nicotine content of tobacco is dependent upon a single factor pair. However, these results do not disprove their contention, for with as variable a factor as nicotine, considerable variation might occur within any one class so that the three classes, homozygous low, heterozygous, and homozygous high, might run together. There are also the complicating factors of nicotine and nornicotine. It will be shown later that in some segregates much of the nicotine seems to change to nornicotine, some of which is determined as nicotine by the A. O. A. C. method.

In 1936 the F_3 generation of some of the very low nicotine plants from the previous year were grown. Of these a few were selected for plant type and nicotine determinations made. The results are given in table 2.

It is evident that burley-colored plants low in nicotine in the F_2 generation do not always produce plants of equally low nicotine content in the F_3 generation; but the F_3 plants from selected low-nicotine F_2 plants are sometimes all relatively low in nicotine and on an average are much lower than burley.

TABLE 2.—*Nicotine content of F_2 plants and of F_3 burleylike plants of low nicotine $\times$ high nicotine hybrids*

F_3 generation of cross—	Nicotine content of F_2 plants	F_3 plants	Range in nicotine content of F_3 plants	Average nicotine content of F_3 plants
	Percent	*Number*	*Percent*	*Percent*
30/33×Ky 7	0.07	4	0.24–.30	0.27
31/33×Ky 14	.01	1	.02	.02
Do	.06	5	1.00–1.56	1.23
Do	.07	5	.32–.98	.70
32/33×Ky 14	.03	5	.46–1.77	1.34
Do	.06	7	.07–1.56	.66
30/33×Ky 7	.01	7	.03–1.11	.60
Do	0	6	1.15–1.78	1.45
32/33×Ky 14	.02	7	.16–.98	.48
Do	.02	9	.52–3.60	1.78
Do	.02	14	.13–2.45	1.26
Do	.02	5	.28–.46	.38

In 1937 determinations of nicotine in seven F_4 plants of a strain from a 0.02-percent F_3 plant showed all to contain 0.05 percent or less; progeny of a 0.07-percent plant contained less than 0.3 percent; progeny of a 0.02-percent plant contained 0.45 percent or less; while in some groups of plants from very low nicotine parents the nicotine content ranged from 0.20 to 2.45 percent. The results seemed to show, however, that extremely low nicotine content strains of large-leafed tobacco having the color of burley could be isolated.

<div align="center">BACKCROSSES</div>

In 1936 it became evident that it would not be possible to select desirable types of tobacco without further backcrossing. Therefore, several backcrosses were made in which Ky 16 was used as the pollen parent and progeny of several very low nicotine plants were used as the seed parents. There was no assurance, however, that the seed parents were all low in nicotine. The next year a good type plant of the F_2 backcross, later shown to contain 1.08 percent nicotine, was crossed with several F_3 32/33×Ky 14, the parent of which tested 0.02 percent nicotine, and with F_4 31/33×Ky 14, of which seven were tested for nicotine and the highest was found to contain 0.05 percent. Another F_2 segregate containing 3.25 percent nicotine was also used (table 3).

TABLE 3.—*Result of crossing F_2 segregates of low-nicotine burleylike plants $\times$ Ky 16, with low-nicotine plants*

Nicotine content of parents (percent)	Plants tested	Nicotine content	
		Range	Average
	Number	*Percent*	*Percent*
0.03 × 1.08	1	0.05	
0.01 × 1.08	19	.01–.60	0.27
0.01 × 1.08	19	.01–.58	.06
0.06 × 1.08	13	.02–1.04	.36
0.13 × 1.08	13	.01–1.21	.21
0.07 × 3.25	17	.05–2.09	1.49
Progeny of 1.08 percent plant used in above crosses	15	.29–.89	.49
0.45-percent ——— selection of 1.08-percent plant	6	.71–1.05	.88
0.33-percent ——— selection of 1.08-percent plant	3	.45–.94	.74

The results of these backcrosses indicate that a plant testing as high as 1.08 percent, when crossed with established low-nicotine strains, produces progeny low in nicotine; but where a high-nicotine plant possibly carrying a factor or factors for low nicotine was used as one parent the F_2 progeny ranged from 0.05 percent to 2.09 percent, with an average of 1.49 percent nicotine.

In 1939, F_3 progeny of plants crossed with the 1.08-percent nicotine plant (table 3) were grown from F_2 plants testing 0.06 percent nicotine or less. Of 52 F_3 plants tested, the nicotine content ranged from 0.01 percent to 2.41, with an average of 0.34 percent. Progenies of several plants were all below 0.1 percent, indicating that very low nicotine strains could be selected following backcrossing with a high-nicotine parent.

In 1940, eight F_4 progeny of a 0.02-percent F_3 plant averaged 0.025 percent nicotine. A selection with 0.9 percent nicotine from the 0.07- $\times$ 3.25-percent nicotine cross (table 3, line 6) gave progeny (seven tested) ranging from 1.06 to 1.59 percent nicotine, with an averge of 1.29 percent, a figure definitely below the nicotine content of burley tobacco grown under the same conditions.

In 1940, the F_2 generation of a second backcross with Ky 16 on a 0.02-percent plant was grown. Nicotine content ranged from 1.11–4.50 in 26 plants tested. While the majority were high, 4 contained less than 1.75 percent nicotine. Four F_3 plants of the plant testing 1.11 ranged from 0.71 to 1.20 percent nicotine.

In 1942, plants from 10 low-nicotine selections from the F_5 generation of crosses listed in table 3 and F_3 second backcrosses of low-nicotine plants of these with Ky 16 were grown. In all, 80 plants were tested for nicotine content. Nicotine ranged from 0.01 percent to 0.90 percent, when Ky 16 grown under the same conditions contained 3.8 percent. The average nicotine content of the 80 plants was 0.246 percent, showing that low-nicotine strains could be isolated after 2 or 3 crosses with burley.

NICOTINE CONTENT OF CROPS OF LOW-NICOTINE TOBACCO

Four small commercial crops of low-nicotine burleylike tobacco were studied for nicotine content in 1945. They were grown on fertile soil and all made rapid, vigorous growth. Nicotine determinations were made of individual seed plants and the nicotine content of a composite sample of a middle leaf from each of 25 topped and suckered plants was determined. A few upper leaves had been stripped from the seed plants, and the plants had been suckered. Samples were collected at the time the remainder of the crop was cut, taking 4 leaves from near the center of the plant from each seed plant and 1 similarly placed leaf from each of 25 topped and suckered plants. Nicotine determinations were made by the quick method of Griffith and Jeffrey (6) after the tobacco was air-cured. Distillations were made from MgO and from NaOH–NaCl.

Three of the strains appear to have been low-nicotine strains (table 4), while one (S 2) apparently contained an occasional plant of higher nicotine content that increased the average nicotine content of this strain above that of the others.

TABLE 4.—*Alkaloid determinations of 4 crops of low-nicotine tobacco, 1945*

Strain	Seed plants	Range in nicotine content determined with MgO	Average	Range in nicotine content determined with NaOH-NaCl	Average
	Number	*Percent*	*Percent*	*Percent*	*Percent*
S 1	25	0.002–0.435	0.109	0.002–0.862	0.231
S 2	24	.001–1.375	.498	.017–1.414	.670
S 3	25	.031–.352	.130	.055–.932	.402
S 4	24	.051–.334	.140	.059–.874	.391
S 1	Composite	.035		.276	
S 2	of 25	.675		.875	
S 3	topped	.173		.489	
S 4	plants.	.125		.427	

A half-acre crop of another strain was grown in 1946 for Dr. H. B. Haag, of the University of Virginia, for his pharmacological studies with low-nicotine tobacco. Dr. Haag reported that after redrying, a thieved sample from a hogshead gave, on analysis, 0.08 percent nicotine, 0.06 percent nornicotine, and 0.14 percent total alkaloids. A previous half-acre crop of low-nicotine tobacco raised for Dr. Haag for studies on the role of nicotine in the cigarette habit contained 0.23 percent nicotine (4).

There seems to be no doubt from these results that commercial crops of low-nicotine tobacco very low in nicotine content can be grown under ordinary field conditions.

NICOTINE AND NORNICOTINE CONTENT OF LOW-NICOTINE TOBACCO

In 1945 and 1946 a modification of the A. O. A. C. method was used for nicotine determinations with the quick distillation apparatus of Griffith and Jeffrey (6). Distillations were made with both MgO and NaOH-NaCl. There is a marked difference in alkaloid content as determined by these methods. So far as Griffith has been able to determine, MgO distillation gives a high value for nicotine since 35 to 40 percent of the nornicotine present in pure or mixed solutions is obtained in the distillate. The NaOH-NaCl concentrations used gave the total steam volatile alkaloids as nicotine. The difference between the MgO and the NaOH-NaCl results gives an approximation of alkaloids other than nicotine, usually assumed to be nornicotine The A. O. A. C. method as used in the early studies (1, *p. 64*), according to unpublished work of Griffith, measures nicotine and from 10 to 30 percent of nornicotine or other compounds and so is not accurate for low-nicotine varieties, some of which appear to contain considerable nornicotine. The present A. O. A. C. (2, *p. 74*) method measures nicotine plus nornicotine and so is not as satisfactory for low-nicotine varieties as the older method.

If the MgO values of all low-nicotine selections are arranged in order, from low to high, and divided by the respective NaOH-NaCl values to determine percentage of alkaloids that is largely nicotine, the figure rises from a very low one (less than 7 percent at the 0–0.01 level) to over 90 percent at the 3-percent level [3] (table 5). That is,

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[3] This relationship was called to the writer's attention by Dr. R. N. Jeffrey.

when nicotine is very low the percentage of total alkaloids that is nicotine is correspondingly low, while as the MgO value rises it becomes a greater and greater proportion of the total alkaloids until in ordinary tobacco 85 to 95 percent of the total alkaloid is nicotine or at least is distilled over from MgO.

TABLE 5.—$\dfrac{MgO}{NaOH-NaCl}$ *values (approximate percent of total alkaloids that is nicotine) in low- and high-nicotine content tobaccos in 1945 and 1946*

MgO value	1945		1946	
	Number of plants	$\dfrac{MgO}{NaOH-NaCl}$	Number of plants	$\dfrac{MgO}{NaOH-NaCl}$
0. – .01	13	0.0660	9	0.0085
.01– .05	14	.2189	23	.0868
.05– .10	26	.3074	13	.1298
.1 – .5	103	.4641	39	.2530
.5 –1.0	44	.5903	14	.6328
1.0 –1.5	27	.8552	10	.8676
1.5 –2.0	27	.8995	2	.8759
2.0 –2.5	8	.8582	--------	----------
2.5 –3.0	2	.8665	--------	----------
3.0 –3.5	6	.9392	2	.8778
3.5 –4.0	1	.9411	--------	----------
4.0 –5.5	3	.9399	--------	----------

Table 5 also shows that the MgO/NaOH-NaCl value, or approximate percent of total alkaloids that is nicotine, in low-nicotine tobaccos was decidedly higher in 1945 than in 1946.

While in general the proportion of nicotine to total alkaloids in the tobaccos studied increases as the total alkaloid content increases (table 5), this does not mean that strains of tobacco cannot be selected that differ markedly in the relation of nicotine to total alkaloids at any given level of the latter. In the progeny of a cross of a 0.02-percent nicotine content burleylike plant × Turkish (Basma) backcrossed with a 0.02 percent burleylike plant, an F_2 of the backcross was selected that tested 0.43 percent nicotine when plants of Ky 16 at the same stage of growth tested 2 percent. The F_3 progeny of this plant ranged in MgO value from 0.082 to 1.007 (average of 24 plants, 0.412), and in NaOH-NaCl value from 0.193 to 1.131 (average of 24 plants, 0.612). The F_4 plants of selected F_3's showed distinct segregation as far as the MgO value was concerned, while the NaOH-NaCl value for the group was remarkably uniform, ranging from 0.38 to 2.23 percent, with 53 of the 75 plants tested lying between 0.85 and 1.8 percent. The range and average of the MgO value, the average of the NaOH-NaCl value, and the ratio between the two values are given in table 6.

In this table the MgO/NaOH-NaCl values have been arranged in descending order, indicating that strains have been isolated having an NaOH-NaCl or total alkaloid content of about 1.5 percent but an MgO value or approximate nicotine content ranging from 94 percent of the total alkaloid content to only 16.6 percent.

TABLE 6.—*Alkaloid content of strains of tobacco resulting from a cross of low-nicotine burleylike tobacco with Turkish tobacco, and Ky 16, as determined by distillation with MgO and NaOH-NaCl, 1946 crop*

Plants (number)	MgO value		NaOH-NaCl average	MgO/NaOH-NaCl
	Range	Average		
	Percent	*Percent*	*Percent*	*Percent*
7	0.58 –1 17	0.89	0.94	0.9408
7	.91 –1.85	1.44	1.59	.9034
3	.19 – .64	.47	⁻.58	.8057
8	.401–1.58	1.06	1.33	.8014
7	.50 –1.10	.77	.98	.7779
4	.18 – .93	.66	.87	.7557
6	.37 –1.50	.94	1.64	.5705
7	.32 – .67	.52	1.21	.4329
9	.11 – .90	.51	1.29	.3945
7	.29 – .71	.49	1.29	.3837
6	.13 – .65	.31	.81	.3830
3	14 – .27	.22	1.32	.1661
Ky 16	3.08 –3.74	3.30	3.68	.8951

While these results are based on small numbers, they give proof that strains of tobacco closely similar to burley in general characteristics can be isolated that have a total alkaloid content of approximately 1 to 1.5 percent when ordinary burley has about 3.5 percent. The results suggest further that in this range strains can be isolated with a large proportion of the alkaloid in the form of nicotine, while other strains appear to have a large proportion of the alkaloid as nornicotine. Strains of cigarette tobacco that are not likely to produce over 1.5 to 2 percent nicotine under conditions that result in 3.5 percent or more nicotine in ordinary burley may prove of great value to the trade.

In 1947 strains derived either directly from the low nicotine × burley backcrosses or from crosses of low nicotine burleylike plants with Turkish tobacco were grown for nicotine determination. The plants from which samples were to be collected were bagged for seed but were kept suckered. Leaf samples were collected from the middle of the plants, at normal cutting time, and air-cured. Nicotine and nornicotine determinations were made by the Bowen-Barthel method (3). Ten plants of Ky 16 and 15 plants each of 19 low nicotine strains were saved for analysis.

The majority of the strains appeared to be fairly homozygous with respect to nicotine and nornicotine. In table 7 the strains are arranged in order of increasing nicotine content. Ten strains proved to be very low in nicotine, the highest averaging 0.05 percent. The other 9 strains ranged from a low average of 0.319 percent nicotine to 0.813. One of the low nicotine strains (range 0 to 0.042) had a range of nornicotine from 0.100 to 1.61 with an average of 0.670. This furnishes the best evidence so far obtained in these studies that strains might be isolated with a relatively high total alkaloid content (about 1 percent) nearly all of which is nornicotine. There is abundant evidence that strains can be isolated with very low nicotine as well as low total alkaloid content.

TABLE 7.—*Nicotine and nornicotine content of 19 strains of low-nicotine tobacco and Ky 16, grown in 1947, as determined by the Bowen-Barthel method*

Nornicotine		Nicotine		Total Alkaloids		Nicotine
Range	Average	Range	Average	Range	Average	Total alkaloids
Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	
0 005– .058	0 028	0 – .006	0. 0004	0. 005– 064	0. 028	. 01
. 242– . 648	. 384	0 – .015	003	. 242– . 648	. 388	008
. 019– . 257	. 138	0 – .069	. 006	. 083– . 257	. 144	. 04
. 026– . 268	. 164	0 – .018	. 007	. 034– . 268	. 162	. 04
. 001– . 062	031	0 – . 027	. 010	. 001– . 077	041	. 24
. 211– . 655	. 412	0 – . 039	. 010	. 230– . 681	. 422	. 02
. 100–1. 61	. 670	0 – . 042	. 013	. 116– . 64	. 683	. 02
0 – . 299	. 049	0 – . 065	. 014	0 – 305	. 063	. 22
. 195– . 582	. 379	0 – . 349	. 044	. 207– . 544	. 422	. 10
. 046– . 201	128	0 – . 254	. 050	. 098– . 300	179	. 28
. 008– 098	052	. 256– . 447	. 319	. 298– . 506	. 371	. 86
. 042– . 373	. 144	. 165– . 615	. 391	. 337– . 832	. 535	. 73
. 035– . 373	. 154	. 137– . 880	. 527	. 280–1 075	. 654	. 81
. 025– . 133	. 074	. 447– . 705	. 589	. 525– . 830	. 656	. 89
. 070– . 253	. 126	. 111– . 855	. 617	. 364–1. 005	. 810	. 76
. 006– . 339	. 139	. 118– . 905	. 642	. 181–1. 174	. 777	. 83
. 043– . 248	. 106	. 381– . 946	. 663	. 556– . 995	. 770	. 86
. 062– . 159	. 122	. 475– . 883	. 690	. 690–1. 022	. 811	. 85
. 040– . 317	. 135	. 457–1. 690	. 813	. 581–1. 852	. 944	. 86
!. 106– . 472	. 290	1. 08 –1. 75	1 457	1. 38–1. 96	1. 760	. 83

[1] Ky.16

When the average nicotine content of each strain was divided by the average total alkaloid content the 19 strains were again divided sharply into 2 groups, one with 28 percent or less of the total alkaloids in the form of nicotine and the other with 73 percent or more of the total alkaloids as nicotine. All of the latter group contained over 0.3 percent average nicotine while all of the former group contained an average of 0.05 percent or less. These low-nicotine strains could all be considered primarily nornicotine strains as most of the alkaloid appears to be nornicotine. Four of the strains in the low-nicotine group contained a higher average total alkaloid content than the lowest of the higher nicotine group. These results suggest that in breeding for low-nicotine varieties of tobacco, 2 sets of factors are concerned: (1) those that control total alkaloid production and (2) those that control the change of nicotine in the leaf to nornicotine or other products of nicotine. It is probably desirable to have varieties in which both sets of factors are operating, if the desired product is to be uniformly low in nicotine year after year. If complete dependence were placed on low total alkaloids these would undoubtedly vary from year to year and the nicotine content might be higher than desirable following, perhaps, a dry year. Moreover, if a factor is operating to change nicotine to some other product, the nicotine content of the tobacco will have much more opportunity to remain constant from year to year.

DISCUSSION

The object in developing a low-nicotine cigarette tobacco was to meet a possible demand for tobacco so low in nicotine that it could be smoked, without harmful effects from nicotine, by people who are sensitive to this alkaloid.

As to the immediate effects of nicotine on the smoker, there are at least two: an increase in blood pressure resulting from absorption of nicotine into the blood stream (7) and irritation of the mucous membranes with which the smoke comes in contact (5). Haag and Larson (8) in preliminary tests showed that—

smoke from the low nicotine cigarettes on an average produced effects comparable to those observed after the smoking of nicotine-free cigarettes, both types of cigarettes evoking circulatory responses, markedly less than those effected by ordinary cigarettes.

Smoke from cigarettes made from low-nicotine tobacco was inhaled by persons with very sensitive throats with only the slightest feeling of irritation, smoke from a low-nicotine cigar with wrapper and binder of the usual type caused distinct but mild irritation, while smoke from an ordinary cigarette caused immediate coughing and left the throat very uncomfortable for several minutes. Cigarettes prepared from low-nicotine tobacco to which about 2 percent nicotine malate had been added (5) affected sensitive throats in exactly the same way as an ordinary cigarette. There seems to be little doubt that the immediate irritation of the throat by cigarette smoke is almost entirely caused by its nicotine content.

While the principal object of the present study was to produce a burley-colored cigarette tobacco of low nicotine content, yet the results have contributed something to the knowledge of the inheritance of nicotine-controlling factors. It seems certain, as has been pointed out by others, that high nicotine is dominant over low nicotine in F_1 crosses. In F_2 crosses the problem of classifying the individual plants is complicated by methods of nicotine determination. The A.O.A.C. (1, 2) method is adapted to high-nicotine varieties in which most of the alkaloid is nicotine so that in the early studies not only nicotine but but nornicotine and perhaps other substances were reported as nicotine. While this did not delay work on selection for low nicotine, it undoubtedly contributed to confusion in understanding results from the genetic standpoint. It is obvious that in the low-nicotine tobaccos the inheritance of at least two sets of factors are involved, those concerned with the rate of nicotine production and those that control conversion of nicotine to nornicotine or other products. If the original alkaloid produced is nicotine, then there must be control mechanisms not only over the rate of nicotine production but also over its conversion to nornicotine, or other products.

The present study has demonstrated that the ability to produce only a small amount of nicotine and a low total alkaloid content may be transferred from low-nicotine cigar tobacco to a burleylike variety of tobacco. Assuming that nicotine is the alkaloid produced by the roots of these strains, then in some strains of low-nicotine tobacco there must be a mechanism for changing nicotine to some other product, presumably nornicotine, while, in other strains this ability occurs only to a very slight extent. If the ability to convert a considerable portion of nicotine to some other product can be transferred to ordinary varieties of high-nicotine content tobacco without disturbing the variety too much otherwise, it is possible that the nicotine content of ordinary tobaccos might be kept at a more desirable level year after year.

The low-nicotine burleylike varieties of tobacco described herein have been recognized by the United States Department of Agriculture (*11*) as a subtype of burley and have been designated Type 31-V.

SUMMARY

Varieties of burleylike tobacco of very low nicotine content have been developed by crossing and backcrossing low-nicotine cigar tobacco with burley. The low-nicotine burleylike tobacco has been recognized by the United States Department of Agriculture as a subtype of burley and has been designated Type 31–V.

First generation crosses between burley and low-nicotine cigar tobacco were high in nicotine. In the F_2 generation there was segregation with respect to nicotine content. The percentage of nicotine ranged from 0 to 2.82, with no sharp line between low- and high-nicotine content. This may have been because both nicotine and nornicotine were reported as nicotine. Progeny of some low-nicotine F_2 plants produced only low-nicotine plants, but this was not always the case.

Commercial crops have been grown with an average nicotine content below 0.10.

In general, when the nicotine content of segregates is very low the percentage of total alkaloids that is nicotine is correspondingly low, while as the nicotine content rises the percentage of total alkaloids that is nicotine also rises until, in ordinary burley tobacco, 85 to 95 percent of the total alkaloid is nicotine. However, evidence was obtained that strains could be isolated with about 1 percent total alkaloid nearly all of which was nornicotine.

When the average nicotine content of low-nicotine strains was divided by the average total alkaloid content 19 strains were divided sharply into 2 groups; one with 28 percent or less of the total alkaloid in the form of nicotine, and the other with 73 percent or more of the total alkaloid as nicotine.

There appear to be two sets of factors controlling the inheritance of nicotine and nornicotine: those that control total alkaloid production, and those that control the change of nicotine in the leaf to nornicotine.

The immediate irritation of the throat by cigarette smoke appears to be caused almost entirely by its nicotine content.

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EFFECT OF HEAT ON BLACK ROT AND KEEPING QUALITY OF SWEETPOTATOES [1]

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INTRODUCTION

Of the many diseases that affect sweetpotatoes (*Ipomoea batatas* (L.) Lam.) in storage, black rot, caused by *Endoconidiphora fimbriata* (Ell. and Halst.) Davidson, probably ranks second in destructiveness only to soft rot caused by *Rhizopus* spp. Very little evidence of black rot may be present at digging time, but a large proportion of the roots may show the disease at the conclusion of storage. Black rot is also a field and seedbed disease, and considerable effort is expended to control it in each of its three phases. The control methods thus far developed for seed potatoes are surface sterilization with mercuric chloride or some similar treatment, careful building up of disease-free seed stock, and rotation of crops. No satisfactory chemical treatment has been devised to prevent the development of black rot in storage or in transit immediately after digging. Discarding visibly affected potatoes does not give control. Washed, freshly dug sweetpotatoes from crops infected with black rot are very often badly spotted by the time they reach the market. A simple and effective method of controlling this disease would be a boon to farmers as well as to storage operators.

Lauritzen [2] determined the range of temperature at which the black rot fungus will grow on culture media to be 49° to 94° F. and showed that infection of sweetpotatoes was possible within about the same range. Since previous experience of the present writers had demonstrated that sweetpotatoes can stand 110° for at least 3 days, the studies reported herein were made to determine the effect of heat treatments on the development of black rot in harvested sweetpotatoes. However, because a treatment that controlled black rot would be of relatively little value if it increased the amount of other rots in the roots, caused excessive loss of weight, reduced the culinary quality, or decreased the sprouting capacity, the effects of the treatments on these factors also had to be evaluated both when black rot was present in the field and when it was absent. In addition, the effect of washing the potatoes on the effectiveness of the treatments had to be taken into consideration.

MATERIAL AND METHODS

Orange Little Stem and Maryland Golden sweetpotatoes were purchased from nearby growers. Except in test 55, all lots were placed under treatment the day they were dug. The material for test 55 was obtained after it had been at a packing shed for an undetermined period. All treatments in tests 52, 55, and 56 had three replicates, but the treatments in tests 53 and 54 were not replicated. Each sample consisted of approximately one-half bushel of sweetpotatoes except in test 54 for which a peck was used.

Three insulated rooms kept, respectively, at approximately 110° F., 85°, and 55° were used. In the 85° and 55° rooms the temperature was held within ±3°, but in the 110° room it ranged from 94° to 114° (fig. 1). The relative humidity was about 80 percent in the 85° and
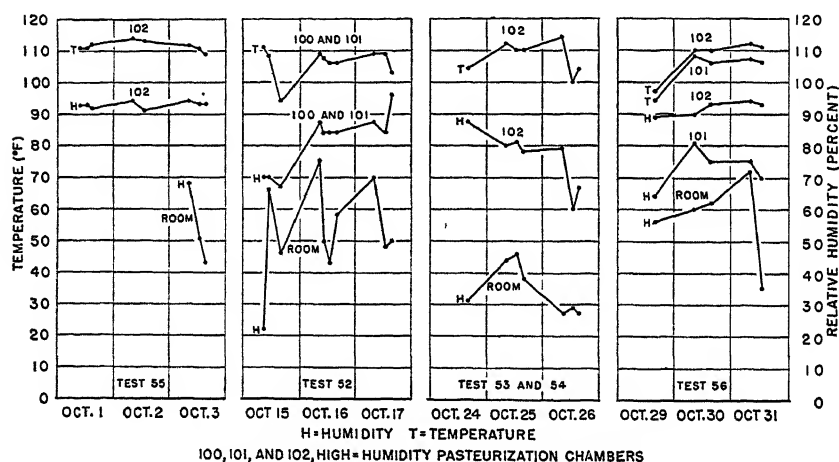
FIGURE 1.—Temperature and relative humidity in the pasteurization room and chambers during the several tests.

55° rooms. In the 110° room two humidity conditions were maintained. In three metal chambers (100, 101, and 102) having a capacity of twelve ½-bushel baskets each and equipped with fans to circulate the air over shallow pans of water, the atmospheric humidity was kept as near 85 percent as possible by regulating the area of exposed water and the rate of air movement over it. Outside the chambers the atmospheric humidity was in general kept at 70 percent or lower by use of anhydrous calcium chloride in shallow pans. The 110° room was designated the pasteurizing room; the 85° room, the curing room; and the 55° room, the storage room.

The term "pasteurization" is used to mean the holding of the sweetpotatoes at 110° F. for various lengths of time. Different lots were pasteurized for 1, 2, or 3 days, some in the chambers (high humidity) and others outside (low humidity).

Curing consisted in holding the sweetpotatoes in the 85° F. room at about 80 percent humidity for 3 or 10 days with or without previous pasteurization.

Each basket was given the number of the test to which it was assigned (52 to 56) and a letter to designate the treatment (table 1).

TABLE 1.—*Description of treatments given sweetpotatoes*

Treatment designation	Pasteuri-zation period	Humidity during pasteuri-zation	Length of curing period	Treatment designation	Pasteuri-zation period	Humidity during pasteuri-zation	Length of curing period
	Days		*Days*		*Days*		*Days*
A (check)	0		10	Pasteurization at 110° F.—Continued			
Pasteurization at 110° F.:							
B	1	High	10	G	3	Low	10
C	1	Low	10	H	1	High	0
D	2	High	10	I	2	do	0
E	2	Low	10	J	2	do	3
F	3	High	10	K	2	Low	3

After the curing period all lots were moved into the storage room' where they remained until they were examined.

RESULTS

SWEETPOTATOES FROM A FIELD FREE OF BLACK ROT

The variety Orange Little Stem from a field free of black rot was used in test 52. The results given in table 2 show the general effects of the treatments on loss of weight through transpiration and respiration during pasteurizing, curing, and subsequent storage for 10 weeks; also, the percentage of sweetpotatoes (on basis of original weight) that remained healthy or affected with soft rot,[3] end rot caused by *Fusarium*, and other rots. No black rot developed in this test.

TABLE 2.—*Effect of pasteurizing and curing treatments on Orange Little Stem sweet-potatoes not infected with black rot and stored for 10 weeks*

[Average of 3 replicates]

Test and treatment [1]	Original weight per half-bushel lot	Weight loss during—			Roots sound [2]	Weight loss from—		
		Pasteur-izing and curing	Storage	Pasteur-izing, curing, and storage		Soft rot (*Mucor* type)	End rot	Other rots
	Pounds	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
52A	27.4	4.4	8.6	13.0	85.2	0.7	0.9	0.0
52B (101)	23.5	5.8	8.4	14.2	79.6	2.1	2.0	.3
52C (room)	27.4	6.2	7.7	13.9	80.1	3.5	1.6	.5
52D (100)	23.1	8.1	7.1	15.2	78.8	3.2	.7	1.9
52E (room)	27.2	6.6	7.9	14.5	75.6	5.7	2.9	.7
52F (100)	23.5	9.1	7.5	16.6	70.9	9.5	1.7	.3
52G (room)	26.1	9.0	7.3	16.3	71.4	8.8	.8	1.0
52H (101)	23.6	1.1	18.8	19.9	41.0	9.2	27.8	1.3
52I (101)	22.7	3.5	16.6	20.1	24.1	25.7	28.6	.9
52J (100)	23.5	5.8	9.0	14.8	68.8	3.3	10.3	2.2
52K (room)	27.3	4.4	8.0	12.4	73.8	7.2	3.7	2.0

[1] See table 1 for details of treatments; 100 and 101 designate high-humidity pasteurization chambers.
[2] Difference required for significance at 5-percent level, 9.4 percent. The statistical analysis was made without converting percentages to angles, because the percentage figures ranged between 20 and 90.

[3] Soft rot in this paper refers to a soft, watery rot similar to that developing at low temperatures and probably caused by an organism of the *Mucor* type. A study of the rot organism is being made.

In this test at 110° F. the relative humidity in the chambers (high) fluctuated around 80 percent and in the room (low) around 60 percent (fig. 1). Under these conditions weight loss and rot development were sufficiently similar for comparable treatments. Loss in weight increased during pasteurization as well as during curing, but subsequently it tended to become equalized during storage for all cured lots. Although the check (treatment A) showed the greatest percentage of healthy stock when examined January 15, this lot was not significantly better than those pasteurized 1 or 2 days. Lack of significance for treatment E, however, is questionable. The 3-day pasteurization caused a material reduction in keeping quality. Pasteurization at 110° for 1 or 2 days without curing was not effective; other lots with only a 3-day curing after 2 days of pasteurizing kept nearly as well as those similarly pasteurized but cured a full 10-day period.

The capacity of these sweetpotato roots to produce sprouts, or draws, together with that of those in test 56 (p. 189), is shown in table 3. The roots in test 52 were bedded January 16 in moist sand in shallow boxes and placed under artificial light in the room originally used for curing (85° F. and 80 percent relative humidity). The number of sprouts that developed was determined February 2. After this period of 17 days under good conditions most of the sprouts above the sand were several inches long.

Table 3 shows that 1 day of pasteurizing followed by 10 days of curing did not lessen the production of sprouts in comparison with curing only; but pasteurizing for 2 or 3 days caused a reduction in sprout production whether the roots were cured 3 or 10 days. Whether this situation would hold true at normal bedding time is not known.

To test the effect of the pasteurizing treatment on culinary quality, five representative sweetpotatoes from each of the treatments were baked and tasted. A bitter or off flavor was found in roots given treatments D, E, F, G, I, and K; those given treatments A, B, and C were judged satisfactory and those given treatments B and C less so than those given A.

TABLE 3.—*Sprout production by pasteurized and nonpasteurized roots of Orange Little Stem and Maryland Golden sweetpotatoes*

Variety, test, and treatment [1]	Weight of 9 roots bedded	Sprouts produced—			Roots dormant	Roots rotted
		Above sand	Below sand	Total		
Orange Little Stem:	*Pounds*	*Number*	*Number*	*Number*	*Number*	*Number*
52A	2.3	99	118	217	0	0
52B	2.0	74	138	212	0	1
52C	2.1	114	156	270	0	0
52E	2.0	43	75	118	0	3
52G	2.0	54	93	147	0	4
52K	2.0	27	77	104	0	1
Maryland Golden:						
56A	2.5	63	33	96	0	0
56B	2.6	71	37	108	0	0
56C	2.4	38	130	168	0	0
56D	2.5	41	24	65	0	3
56E	2.7	49	40	89	2 1	0
56G	2.9	57	56	113	0	1

[1] See table 1 for details of treatments.
[2] Partly rotted on each end.

COMMERCIALLY WASHED SWEETPOTATOES THAT CONTAINED SOME
BLACK ROT AT DIGGING

Because of their similarity in material, methods, and results, tests 53 and 55 are considered together. Test 53 was started October 24 and test 55 October 1, and the roots were examined January 7 and 8, respectively. Maryland Golden sweetpotatoes that had been washed commercially were used in both tests. The roots used in test 53 were placed under test conditions the day they were dug; those in test 55 were obtained from a packing shed where they had been for an undetermined length of time after digging. Both lots contained some black rot when delivered to the shed. All visibly infected potatoes were removed after the roots were washed. In all check lots (A) some black rot developed during the storage period, but not a single black rot lesion could be found in any of the pasteurized lots (table 4). This indicates that sweetpotatoes infected with black rot or carrying black rot inoculum can be effectively pasteurized and that this disease can be arrested by subjecting the roots to 110° F. for 1 day. The effect of the pasteurizing treatment on the development of other rots is not clear.

TABLE 4.—*Kind and amount of decay developing in pasteurized and nonpasteurized washed Maryland Golden sweetpotatoes that were cured*

| Test and treatment [1] | Total roots tested | Roots sound | Roots affected with— | | | | | | Mum-mies |
			Black rot	Soft rot (Mucor type)	End rot	Surface rot [2]	Other rot	
	Number	Number	Number	Number	Number	Number	Number	Number
53A	68	32	18	14	3	0	0	1
53B (102)	24	24	0	0	0	0	0	0
53C (room)	36	24	0	9	3	0	0	0
53D (102)	41	25	0	1	15	0	0	0
53E (room)	44	11	0	1	31	0	1	0
55A [3]	311	28	23	174	38	1	0	47
55B (102) [3]	137	56	0	17	34	22	0	8
55C (room) [3]	134	32	0	41	25	3	0	33

[1] See table 1 for details of treatments; 102 designates a high-humidity pasteurization chamber.
[2] Surface rot could easily have been classed as desiccated skinned areas or bruises on healthy potatoes.
[3] 55A represents 6 replicates; 55B and 55C, 3 replicates each.

SWEETPOTATOES WITH BLACK ROT LESIONS PRESENT

In test 54 an attempt was made to kill the black rot organism after it was well established. On Maryland Golden sweetpotatoes that had black rot lesions at the time of digging the margins of the lesions were carefully outlined with an indelible pencil before the roots were treated. About 1 peck of such sweetpotatoes was used for each treatment. This test was begun October 24 and the roots were examined January 7, when they were divided into three classes: (1) Those on which the lesions had enlarged beyond the indelible line; (2) those on which the lesions had not enlarged beyond that line; and (3) those so badly rotted by other organisms that it was impossible to tell what had happened to the black rot lesions (table 5 and fig. 2) On only one root from the pasteurized lots did rot develop beyond the indelible line, and it was doubtful whether black rot was the cause of this

FIGURE 2.—*A*, Black rot lesions on sweetpotatoes pasteurized 1 day, which did not enlarge; *B*, lesions on nonpasteurized sweetpotatoes, which enlarged during storage.

extension, because isolations from it did not yield the black rot fungus. When isolations were made from the enlarged lesions on the check lots, however, several cultures of the black rot fungus were obtained; but none was obtained from the lots pasteurized for 1 day. It was noted also that the lesions on pasteurized roots remained shallow like those on freshly dug potatoes, whereas some of the enlarged lesions on the check lots had progressed into the tissue of the potato for as much as half an inch. Some of the other lesions did not develop beyond the stage they had reached at the time of treatment. These were shallow and appeared to be inactive or dead, like those on the pasteurized lots, indicating that some of the black rot lesions found at digging do not remain active.

TABLE 5.—*Enlargement of black rot lesions on pasteurized and nonpasteurized roots of Maryland Golden sweetpotatoes during 10 weeks in storage*

Test and treatment [1]	Potatoes tested	Margins of lesions extended	Margins of lesions not extended	Lesions obscured by other rots
	Number	*Number*	*Number*	*Number*
54A–1	16	[2] 7	3	6
54A–2	17	[2] 5	6	6
54B (102)	16	0	[3] 13	3
54C (room)	16	[3] 1	[3] 8	7
54D (102)	18	0	15	3
54E (room)	15	0	8	7
54F (102)	15	0	5	10
54G (room)	15	0	10	5

[1] See table 1 for details of treatments; 102 designates a high-humidity pasteurization chamber.
[2] Isolations yielded black rot fungus.
[3] Isolations did not yield black rot fungus.

UNWASHED SWEETPOTATOES THAT CONTAINED SOME BLACK ROT AT DIGGING

In test 56, started October 29 and examined January 8, Maryland Golden sweetpotatoes from a field where black rot was in evidence were put through the same series of pasteurizing and curing. Careful records were made of weight losses and types of rots that developed. All roots having black rot lesions at time of digging were sorted out and thrown away. Unlike the sweetpotatoes in tests 53 and 55, these were not washed. Although these roots came from a field where only a few roots were infected with black rot, in the three check baskets an average of 8 percent by weight of the original lot developed the disease (table 6). The percentages of sound roots except after the 3-day pasteurization, while not greatly increased in the pasteurized lots, indicate that had the black rot taken a greater toll the pasteurized lots would have had considerably more sound roots; or, if the surface rots had been considered as desiccated bruises and classed as sound, all the pasteurized lots, except those given treatment G, would have had considerably larger percentages of healthy roots. Results for treatment F are omitted from the table because the 3 days at high humidity and 110° F. induced to a marked degree a type of decay occasionally noticed in similarly treated roots in the other tests— that is, a shallow, pitlike rot probably caused by a species of *Rhizopus*. In treatment 56F this did not stop and dry up as in the other lots pasteurized 3 days but continued until nearly every infected potato was destroyed.

TABLE 6.—*Effect of pasteurizing and curing treatment on Maryland Golden sweet-potatoes infected with black rot and stored for 10 weeks*

[Average of 3 replicates]

Test and treatment [1]	Original weight per half-bushel basket	Weight loss during—			Roots sound	Weight loss from—			
		Pasteurizing and curing	Storage	Pasteurizing, curing, and storage		End rot	Surface rot [2]	Black rot	Other rots
	Pounds	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
56A_____	22.3	3.5	2.7	6.2	76.5	5.4	2.9	8.0	0.0
56B (102)_____	21.7	5.4	3.7	9.1	77.7	4.1	7.6	.0	.8
56C (room)_____	20.0	5.5	3.7	9.2	84.2	2.3	2.9	.0	1.0
56D (102)_____	20.8	7.0	2.8	9.8	85.4	1.9	2.6	.0	.0
56E (room)_____	21.7	7.4	3.1	10.5	76.9	3.4	5.8	.0	3.4
56G (room)_____	22.2	8.3	4.5	12.8	74.2	1.5	6.5	.0	5.1

[1] See table 1 for details of treatments; 56F was discarded November 13, 1947, because of excessive rotting; 102 designates a high-humidity pasteurization chamber.
[2] Surface rot could easily have been classed in most cases as desiccated skinned areas or bruises on healthy potatoes.

In considering the effect of these pasteurizing treatments upon the development of other rots it was noted that prolonged treatment of potatoes was detrimental, and that the type of rot that developed seemingly depended on factors other than the pasteurization treatment itself.

Some roots from each of these treatments were bedded January 12 and were examined for sprouts February 2. The results indicate again that pasteurization for 1 day did not hurt the sprouting capacity

of the potatoes (table 3). However, these data are not so clear-cut as those for Orange Little Stem (table 3). Irregularity of sprouting was also noted in other tests with Maryland Golden.

SUMMARY AND CONCLUSIONS

In sweetpotatoes freshly dug from fields where black rot occurred subjection to 110° F. for 1 day or more and then curing at 85° and 80 percent humidity prevented the development of black rot in storage and killed the causal fungus on lesions present when dug. In roots treated for more than 1 day at this temperature an off flavor was found in baked lots, sprout production was inhibited, and frequently certain other types of decay were prevalent. If treated for only 1 day at 110° the sweetpotatoes were not materially damaged in any way.

A FURTHER TOXICOLOGICAL COMPARISON OF DERRIS AND LONCHOCARPUS [1]

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In a recent article [2] a report was made of a toxicological comparison of some authentic samples of roots of *Derris* and *Lonchocarpus* varieties. In the work therein described several types of *Derris* and *Lonchocarpus* were used, each of which was represented by one or two samples of roots grown in the same location. The report showed derris root to be generally superior to lonchocarpus root even when the rotenone content was equal. The present report deals with a similar comparison of the same species with some of the types represented by samples grown under different conditions. Additional chemical analyses were made in an attempt to trace the toxicity due to compounds other than rotenone.

The varieties of *Derris elliptica* (Roxb.) Benth. used were: Sarawak Creeping, St. Croix, and Changi No. 3. The Sarawak Creeping variety was represented by samples of roots grown in Puerto Rico and of roots grown in Guatemala. The Changi No. 3 was represented by samples of the Rio Piedras clone and the Mayagüez-Goodyear (MG) clones. The lonchocarpus root used was of *Lonchocarpus utilis* A. C. Smith and *L. chrysophyllus* Kleinh. Of *L. utilis,* one sample was from plants grown in Puerto Rico and one from plants grown in South America. This gave a set of 8 root samples, each of which represented 10 or more plants.

The toxicological tests were made on houseflies (*Musca domestica* L.) and Mexican bean beetles (*Epilachna varrivestis* Muls. The samples for the tests with houseflies were prepared as in the previous study,[3] and the dilutions used were such that 1 ml. of the test solutions contained 0.09 mg. of rotenone. The tests were carried out by the turntable method on adult houseflies reared by standard procedure. Eight tests were made on each sample with about 150 flies to each test. For reference, tests were also made with solutions of 5 graded concentrations of pure rotenone. The toxicity of the rotenone standards was plotted against concentration on log-probability paper and a straight line fitted. The rotenone equivalents of the root samples were then obtained by reference to this graph. The results are given in table 1.

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TABLE 1.—*Comparative toxicity of some derris and longchocarpus samples to house-flies*

Sample and where obtained	Concentration [1]	Mortality of flies in 3 days [2]	Rotenone equivalent of test solution	Rotenone equivalent of root	
				Total	Rotenoids
	Mg./ml.	*Mean percent*	*Mg./ml.*	*Percent*	*Percent*
D. elliptica:					
Sarawak Creeping, Puerto Rico___	1.69	46	0.222±0.015	13.14±0 89	7.74
Sarawak Creeping, Guatemala____	1.71	33	.146± .011	8.59± .64	3.29
Changi No. 3, MG_____	1.25	.35	.153± .012	12.24± .92	4.94
Changi No. 3, Rio Piedras_____	2 44	35	.153± .012	6.24± .49	2.54
St. Croix_____	4.99	48	.236± .016	4.73± .32	2.93
L. utilis:					
Puerto Rico_____	1.64	32	.140± .011	8.54± .67	3.04
South America_____	1.91	37	.171± .013	9.00± .68	4.30
L. chrysophyllus_____	1.55	23	.106± .009	6.84± .58	1.14
	1	23	.100± .009	------------	----------
	.2	40	.200± .013	------------	----------
Rotenone_____	.4	68	.400± .034	------------	----------
	.8	81	.800± .070	------------	----------
	1 6	91	1.60 ± 23	------------	----------

[1] Concentration of oven-dry, powdered root, the concentration of rotenone in each test solution was 0.09 mg. per milliliter.
[2] Standard error of mean mortalities is 2.2 percent. Differences between mean mortalities greater than 6 percent are significant at adds of 19:1.

For the tests on the Mexican bean beetle, the spray tower-petri dish technique was used. This method was designed to duplicate some of the practical conditions that would be encountered in control work in the field. Excised bean leaves with petioles in vials of water were treated uniformly on both sides by a falling mist spray consisting of a suspension of the powdered sample in water containing acacia. The amount of sample in the spray was adjusted for each sample so that the rotenone concentration was 0.0125±0.0002 percent. To prepare the control samples, an acetone solution of rotenone was mixed thoroughly with fuller's earth and the acetone evaporated. The powder was then passed through a 200-mesh sieve and suspended in water containing the wetting agent. As soon after the spraying as the water had evaporated from the surface the leaves were placed in 6-inch petri dishes and infested with 10 third instar larvae of the Mexican bean beetle. The larvae were confined in the dish by a 16-mesh screen cover. After 3 days the treated leaves were removed and an unsprayed leaf placed in each dish. This was done to permit larvae that were only slightly poisoned by the spray to feed on fresh foliage and recover. Thus those repelled by the spray deposit would not starve and so give a false idea of the value of the insecticide. It was felt that this practice resembles practical conditions under which, about 3 days after spray treatment, there is usually untreated leaf area present on bean plants, due to new growth, opening of flowers, or removal of spray deposit by weathering. However, the technique differed from practical conditions in that the spray was not applied directly to the insect as it would be to a great extent in spraying a bean field. Also under practical conditions larvae only slightly poisoned may fall off the plant and be killed by predators, trampling, or desiccation. Therefore, under the conditions of the tests the larvae had a better opportunity to recover from slight poisoning.

The spray residue may have acted as a contact insecticide on the insects that crawled on it. It also acted as a stomach poison when the leaf was fed upon by the larvae. The dosage received by a larva was also probably affected by the repellent action of some constituents of the spray. Final mortality counts were made on the sixth day. The entire series was run in duplicate daily for 10 days to give a total of 20 tests for each of the 8 samples and 2 controls. In order to determine how much of the leaf was fed upon, the area eaten was estimated by placing a grid on the leaf and counting the number of square centimeters missing. Where only portions of a square centimeter were eaten, the area was estimated. The results of the tests are given in table 2.

TABLE 2.—*Comparative toxicity of some derris and lonchocarpus samples to Mexican bean beetle larvae*

Sample and where obtained	Concentration in spray	Amount eaten [1]	Mortality in 6 days [2]	Calculated mortality per cm.[2] consumed	
				Rotenone [3]	Total root [4]
D. elliptica:	*Gm./100 ml.*				
Sarawak Creeping, Puerto Rico	0. 23	*Cm.[2] test* 1. 6	*Percent* 49	*Percent* 31	*Percent* 33
Sarawak Creeping, Guatemala	. 24	3. 2	42	13	14
Changi No. 3, MG	. 17	1. 9	37	19	28
Changi No. 3, Rio Piedras	. 34	2. 0	37	18	13
St. Croix	. 69	2. 1	44	21	8
L. utilis:					
Puerto Rico	. 23	4. 3	27	6	7
South America	. 27	4. 1	39	10	9
L. chrysophyllus	. 22	6. 3	32	5	6
Rotenone preparation [5]	. 25	5. 0	40	8	8
Rotenone preparation [6]	. 25	3. 8	38	--------	10

[1] 5.0 cm.[2] is a significant difference.
[2] 10 percent is a significant difference.
[3] Equal dosages of rotenone.
[4] Equal dosages of root (0.25 gm. per 100 milliliter).
[5] Standard 5 percent rotenone in fuller's earth.
[6] Standard 20 percent rotenone in fuller's earth.

Chemical examination of the samples consisted of rotenone analyses by the method of the Association of Official Agricultural Chemists.[4] The procedure outlined by Goodhue and Haller [5] was used to determine the alkali-soluble fraction, fats and waxes, and the neutral resin. The content of rotenone plus rotenoids was determined by a

[4] ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. OFFICIAL AND TENTATIVE METHODS OF ANALYSIS . . . Ed. 5, 757 pp., illus. Washington D. C. 1940. (See pp. 64–66.)
[5] GOODHUE, L. D., and HALLER, H. L. THE NON-CRYSTALLINE CONSTITUENTS OF TEPHROSIA VIRGINIANA ROOTS. Amer. Chem Soc. Jour. 62: 2520–2522. 1940.

colorimetric procedure [6] and deguelin by the method of Goodhue and Haller.[7]

The tests with houseflies allow the samples to be ranked as follows with regard to toxic value: (1) Derris Sarawak Creeping from Puerto Rico and derris Changi No. 3, MG clone, (2) the two *Lonchocarpus utilis* samples and the derris Sarawak Creeping from Guatemala, (3) *L. chrysophyllus* and derris Changi No. 3, Rio Piedras clone, and (4) derris St. Croix.

The mortality of larvae on the foliage, treated or untreated, varied considerably. In trials with untreated foliage, 8 tests early in the period of testing gave 2 percent average mortality, 6 tests near the end of the period gave 40 percent, and 16 tests in the final week gave 72 percent. The data for rotenone in fuller's earth show clearly that the toxicity was not proportional to the rotenone content of the spray; a 5-percent rotenone preparation gave the same kill as a preparation containing 20 percent. Likewise, in tests not shown in table 2, 1.25 percent rotenone in fuller's earth gave 62 percent kill, while a preparation in which pure rotenone was used gave only 70 percent kill. From these results it is apparent that little increase in effectiveness resulted from increasing the concentration of rotenone in the spray. The larvae apparently compensated for the increased rotenone by reducing their feeding and contact. That the amount of feeding decreased as the concentration of rotenone was increased was shown by the following tests which were made in pairs: With 1.25 percent rotenone, 6.0 cm.^{2} was eaten, and with 98 percent, 1.2 cm.^{2}; with 2.5 percent, 14.1 cm.^{2}, and with 10 percent, 9.1 cm.^{2}; with 5 percent, 5 cm.^{2}, and with 20 percent, 3.8 cm.^{2}. Therefore, since it was not possible to arrive at rotenone equivalents as was done with the data for houseflies, the comparison was made on the basis of mortality in 6 days with equal rotenone dosages and the amount of feeding was taken into account.

The data indicate that the derris samples caused higher mortality than the lonchocarpus and permitted less feeding when equal dosages of rotenone were used. On the basis of mortality per unit area consumed the Puerto Rican Sarawak Creeping and the Changi No. 3 MG roots appear to be the most effective and the St. Croix derris and the lonchocarpus samples the least.

The rotenone equivalents calculated from the toxicological tests on houseflies are shown in table 3, together with the results of chemical examination.

Since it was not possible to calculate rotenone equivalents from the data with the bean beetle, only the data from the tests with houseflies are included for comparison with those obtained by chemical examination. Such comparison brings out several points. It shows (1) that deguelin was not the only toxicant besides rotenone and (2) that the

[6] Jones, M. A. the application of a modified red-color test for rotenone and related compounds to derris and lonchocarpus. Assoc. Off. Agr. Chem. Jour. 28: 352–359, illus. 1945.
[7] Goodhue, L. D., and Haller, H. L. a method for determining deguelin in derris and cube. Indus. and Engin. Chem., Analyt. Ed. 11: 640–642. 1939.

alkali-soluble fractions and the fats and waxes had no appreciable toxicity. The compounds that contribute the extra toxicity apparently occur in the neutral resin fraction. Toxicity over that due to rotenone was roughly proportional to the neutral resin content. The percentages of neutral resin indicate that it contained varying amounts of toxicant.

TABLE 3.—*Chemical analysis and rotenone equivalent of some derris and lonchocarpus samples as determined by tests on houseflies*

Sample		Chemical analyses (dry basis)									Rotenone equivalent of roots
Type	Where grown	Plants represented	Total extractives	Rotenone plus rotenoids	Rotenone	Alkali-soluble fraction	Fats and waxes	Neutral resins	Deguelin	Toxicarol and tephrosin	
		Number	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
D. elliptica:											
Sarawak Creeping.	Puerto Rico	162	16.4	12.7	5 4	0.32	1.41	10 49	0 16	0.12	13 14
Do.	Guatemala	80	12.6	9.9	5.3	.33	.89	7.08	------	1 05	8 59
Changi No. 3, MG.	Puerto Rico	191	16 6	13.5	7 3	.48	2 45	7.31	22	1.29	12.24
Changi No. 3, Rio Piedras.	do	129	7.8	6.3	3.7	.34	1.43	3 86	------	75	6 24
St. Croix.	do	854	5 9	4.6	1 8	.37	1.13	3.81	.13	78	4.73
L. utilis.	do	10	8.3	6.4	5.5	.22	1 97	2 43	------	.05	8.54
Do.	South America.	32	12.4	8 8	4 7	.67	1.73	4.02	2.64	3 27	9.00
L. chrysophyllus	Puerto Rico	10	7 7	6 2	5 7	.37	1.05	2 06	------	.32	6.84

Inasmuch as such roots are valued for their insecticidal action and accurate biological tests are expensive on a large scale, it may be of value to rank the samples according to toxicity and compare this order with that obtained by use of the chemical analytical criteria. Such a comparison is shown in table 4.

From this table it can be seen that ranking according to the content of rotenone plus neutral resin or according to total chloroform extractives agrees most closely with the order obtained by tests with houseflies. Neither of these criteria gives numerical values which agree even closely with the numerical values for rotenone equivalent; for numerical agreement the colorimetric determination for rotenone plus rotenoids gives the best agreement but it fails to bring out small differences.

With regard to the two types represented by samples grown in different localities, it is clear that the toxicity per unit of rotenone content was considerably different in the same variety grown in two localities; this indicates that growing conditions, as well as variety, affect the toxicity of the nonrotenone constituents of the roots.

TABLE 4.—*Rank of samples according to several criteria* [1]

Sample and where obtained	Rotenone equivalent	Rank based on—				
		Toxicity to houseflies	Total chloroform extractives	Rotenone plus rotenoids	Rotenone	Rotenone plus neutral resin
D. elliptica:	*Percent*					
Sarawak Creeping, Puerto Rico	13. 14	1 or 2	1 or 2	2	2, 3, 4, or 5	1
Changi No. 3 MG	12. 24	1 or 2	1 or 2	1	1	2
L. utilis:						
South America	9. 00	3, 4, or 5	3 or 4	4	6	4
D. elliptica:						
Sarawak Creeping, Guatemala	8. 59	3, 4, or 5	3 or 4	3	2, 3, 4, or 5	3
L. utilis:						
Puerto Rico	8. 54	4 or 5	5	5, 6, or 7	2, 3, 4, or 5	5, 6, or 7
L. chrysophyllus	6. 84	6 or 7	6 or 7	5, 6, or 7	2, 3, 4, or 5	5, 6, or 7
D. elliptica:						
Changi No. 3, Rio Piedras	6. 24	6 or 7	6 or 7	5, 6, or 7	7	5, 6, or 7
St. Croix	4 73	8	8	8	8	8

[1] Note that the rank is not based on a uniform scale but rather on relative order among the 8 samples, 1=highest, 8=lowest.

SUMMARY

Nine chemical characteristics and the toxicity to two species of insects were determined for eight samples of derris and lonchocarpus root. When the samples were ranked on the basis of toxicity, rotenone plus natural resins and total chloroform extractives were found to be most nearly in accord with their toxicity to houseflies. The rotenone plus rotenoids ranking was fairly similar to the insecticidal ranking; rotenone content as well as the other chemical characteristics determined showed considerable divergence from the toxicological value.

In general, derris had a higher insecticidal value than lonchocarpus when comparisons were based on equal rotenone content. The derris grown in Puerto Rico was much more toxic than that grown in Guatemala, but there was little difference in the lonchocarpus samples from Puerto Rico and South America.

The housefly was more satisfactory than the larvae of the Mexican bean beetle as a test insect for evaluation of the relative toxicity of these samples. However, despite the dissimilarity in the manner in which the insecticides were applied, the rankings according to toxicity to the two species agreed when the differences between samples were relatively great.

COMPARISON OF CHEMICAL VALUES WITH THE TOXICOLOGICAL ROTENONE EQUIVALENT OF DERRIS AND LONCHOCARPUS ROOTS [1]

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INTRODUCTION

During the recent war rotenone was an important item on the list of scarce and strategic materials of the United States. The main source of supply was cut off when the Japanese invaded the principal derris-producing areas of the world. As a result, large quantities of *Lonchocarpus* or cube roots were imported from Peru and other South American countries, where this rotenone-bearing species grows extensively. Table 1 shows the relative percentages of derris and

TABLE 1.—*United States imports of derris and lonchocarpus roots, from 1941 to 1946* [1]

Species	1941	1942	1943	1944	1945	1946 [2]
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Lonchocarpus root	48 7	73.0	100	100	99.4	99.8
Derris root	51.3	27.0			.6	.2

[1] From a report by Lawrence Witt (*10*), Office of Foreign Agricultural Relations, U. S. Department of Agriculture.
[2] Data for 1946 from Claud L. Horn of the same office.

lonchocarpus root imported into the United States between 1941 and 1946.

Assuming that the inventories of most importers are small from year to year, it is evident from these data that practically the entire amount of rotenone used in the United States during the past 4 years was obtained from the roots of lonchocarpus. The results of a field experiment performed by Dr. G. E. R. Hervey of the New York State Agricultural Experiment Station [2] indicate that insecticidal preparations made from derris may be more toxic than those made from lonchocarpus at the same rotenone level. In Hervey's experiment samples of powdered derris and lonchocarpus root each containing 5 percent rotenone were diluted with talc to make dusts containing 1.0, 0.5, and 0.25 percent rotenone. These dusts were applied to cabbage having a light infestation of the imported cabbageworm (*Ascia rapae* (L.)) and cabbage looper (*Autographa brassicae* (Riley)).

[1] Received for publication June 25, 1948.
[2] Unpublished data.

The dusts were applied three times at an average rate of 25 pounds per acre with an interval of about 2 weeks between applications. The results, which are shown in table 2, are based on the quantity of foliage

TABLE 2.—*Comparative control of the imported cabbageworm and cabbage looper with rotenone dust from derris and lonchocarpus roots* [1]

Source	Rotenone	Average injury index	Control
	Percent	*Percent*	*Percent*
Derris	1.0	17.1	70.2
Lonchocarpus	1 0	21.5	62.5
Derris	.5	22.1	61.5
Lonchocarpus	.5	25.0	56.4
Derris	.25	29.1	49.3
Lonchocarpus	.25	28.8	49.8
Check		57.4	
Least difference required for significance (19:1)		3.3	

[1] Unpublished data from G. E. R. Hervey, New York State Agricultural Experiment Station.

consumed and were taken when insect activity had ceased. These data show that the insect control obtained with 1.0 percent rotenone dust made from derris root was significantly better than that obained with dust of the same rotenone content made from lonchocarpus. The insect control obtained with 0.5 percent rotenone dust made from derris was better, although not significantly so, than that made from lonchocarpus. The control with 0.25 percent rotenone dust made from derris and lonchocarpus was about the same. In commercial practice these insecticidal plants are evaluated and sold mainly on the basis of their rotenone content, but this criterion apparently does not indicate their toxicological value.

In 1944 Pepper and Filmer (9)[3] reported results of insecticidal tests in which an insecticidal plant of low rotenone content, *Derris malaccensis* Prain, was compared with other species of *Derris* containing a higher rotenone content. Their data showed a higher toxicological value for *D. malaccensis* than for the other species. More recently Jones et al. (5) have presented data which show that "* * * the colorimetric determination of rotenone plus rotenoids was the best chemical estimate of the toxicity of the samples, although it varied considerably from the biological evaluation."

A criterion other than rotenone percentage is needed to establish the true toxicological value of these insecticidal plants. Such a criterion, to be used commercially or when large numbers of samples are involved, must of necessity be simple, rapid, and reasonably accurate. Pagán and Loustalot (8) suggested the use of transmittance values of acetone extracts of derris roots measured in a spectrophotometer at 360 mμ. These measurements correlated fairly closely with the rotenone equivalent as determined biologically. However, this method has certain limitations due to the fact that special apparatus and technique are needed. Likewise, the red-color value, suggested by Jones (4), also has limitations because a colorimeter or other suitable instrument is needed, the conditions required for the test are rather exacting, and the results are not always in close agreement with the biological assays.

[3] Italic numbers in parentheses refer to Literature Cited, p. 204.

Total extractives as a measure of toxicity have not been used extensively because these values are generally higher and thus do not closely approximate the true insecticidal value of the roots. Also, in comparing some of these chemical analyses with results of tests on insects, derris and lonchocarpus samples frequently were included in the same experiments. In this paper data from derris and lonchocarpus samples are presented which show a comparison between some chemical analyses, such as total extractives, red-color values, and rotenone and the rotenone equivalent (determined biologically). In these comparisons the data from derris and lonchocarpus samples are treated separately.

MATERIALS AND METHODS

Thirteen rotenone samples were available for comparison. Eight of these were from roots of *Derris elliptica* and 5 were *Lonchocarpus* (3 of *L. utilis* and 2 of *L. chrysophyllus*) (*5, 6*). All samples were assayed biologically on houseflies (*Musca domestica* L.) by W. A. Gersdorff and E. R. McGovran of the Bureau of Entomology and Plant Quarantine.[4] These 13 samples were used because the essential data were at hand; data on more samples were desired but no other biologically assayed samples were available. Rotenone analyses were made according to the procedure of the Association of Official Agricultural Chemists (*2, pp. 64–66*), and total chloroform extractives were determined from an aliquot of the chloroform extract as obtained in the rotenone determination. The procedure described by Jones (*4*) was used to determine the red-color values. The regression equation and the correlation coefficient between the biologically determined rotenone equivalent and each of the afore-mentioned chemical values were calculated.

RESULTS

Table 3 gives the correlation coefficients and regression equations calculated between the toxicological rotenone equivalent of derris samples and the three chemical values of rotenone, red-color value, and total chloroform extractives. Although all three correlation coefficients were highly significant, the values for total chloroform extractives and red-color value were much higher than that for rotenone, indicating that total chloroform extractives and red-color value are better criteria. The *t* values obtained indicated highly significant regression coefficients in all cases, but again the value for total chloroform extractives (10.19) and for red-color value (10.98) were both considerably higher than the value for rotenone (4.36). The standard error of estimate calculated for the three analytical values show that the error in estimating toxicity from rotenone is about twice that from total chloroform extractives and red-color value.

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[4] Merriam A. Jones, formerly chemist at this station, also participated in the chemical analyses herein reported.

TABLE 3.—*Correlation coefficients, standard error of estimates, and regression equations between toxicological rotenone equivalent and analytical values of some derris and lonchocarpus samples*

Constituent	Correlation coefficient [1]		Standard error of estimate		Regression equations [2]	
	Derris roots	Loncho-carpus roots	Derris roots	Loncho-carpus roots	Derris roots	Lonchocarpus roots
Total chloroform extractives.	0 972	0.435	1 20	1.01	$T=0.932+0.868E$	$T=6.090+0.165E.$
Red-color value	.972	.300	1. 22	1.01	$T=1.0620-0.709C$	$T=6.490+0.1610C.$
Rotenone	877	−.832	2 47	.64	$T=1.471+1.730R$	$T=18.160-1.920R.$

[1] For the derris samples correlation coefficients were highly significant (1-percent level) in all cases, but for the lonchocarpus samples they were not significant.
[2] The regression coefficients of the equation were significant at the 1-percent level for the derris samples, but not significant for the lonchocarpus samples. In these equations $T=$ toxicological rotenone equivalent, $C=$ red-color value, $E=$ total chloroform extractives, and $R=$ rotenone.

The regression lines between the rotenone equivalent of the derris samples and the rotenone content, red-color value, and total chloroform extractives, were plotted (fig. 1) from values obtained from the equations presented in table 4. It is evident from these graphs that the best agreement with rotenone equivalent is obtained with total chloroform extractives, and the next best with red-color value.

TABLE 4.—*Comparison of biological rotenone equivalent of 8 derris samples with that calculated from total chloroform extractives, red-color value, and rotenone content*

Sample No.	Variety	Rotenone equivalent [1]	Total chloroform extractives		Red-color value		Rotenone	
			Actual	Calculated [2]	Actual	Calculated [2]	Actual	Calculated [3]
		Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
D-1	Changi No. 3, MG clone	18.7	22.3	18.4	18.2	18.6	10.0	18.8
D-2	Sarawak Creeping	15.7	16.8	13.7	13 5	13.6	5.7	11.3
D-3	____do____	13.1	16.4	13.3	12.7	12.8	5.4	10.8
D-4	Changi No. 3, MG clones	12.2	16.6	13.5	13 5	13.6	7.3	14.1
D-5	Changi No. 3, P. R. clones	9.4	12.4	9.8	10.2	10.1	5.3	10.6
D-6	Sarawak Creeping	8.5	12.6	10.0	9 9	9 8	5.3	10.6
D-7	Changi No. 3, P. R. clone	6.3	7.8	5.8	6 3	6.0	3.7	7.9
D-8	St. Croix	4.7	5.9	4.2	4.6	4.2	1.8	4.6

[1] Determined biologically on houseflies.
[2] These values are rotenone equivalents calculated from the respective regression equations.

It is interesting to note that if the regression line between rotenone equivalent and rotenone (fig. 1, *A*) is projected until it intersects the abscissa axis a hypothetical root is found with some toxicity and no actual rotenone content. Actually, such a root has been reported by Pepper and Filmer (*9*), who found that a simple of *Derris malaccensis* root had only a trace of rotenone but relatively high toxicological value.

This is additional evidence that at least with derris roots the rotenone content is not a dependable criterion of the insecticidal value of the roots. When the regression line obtained between red-color value and rotenone equivalent was projected (fig. 1, *B*), it intersected the ordinate axis, indicating that a hypothetical case may be found in which there is some red-color value but no toxicity. No instance of this
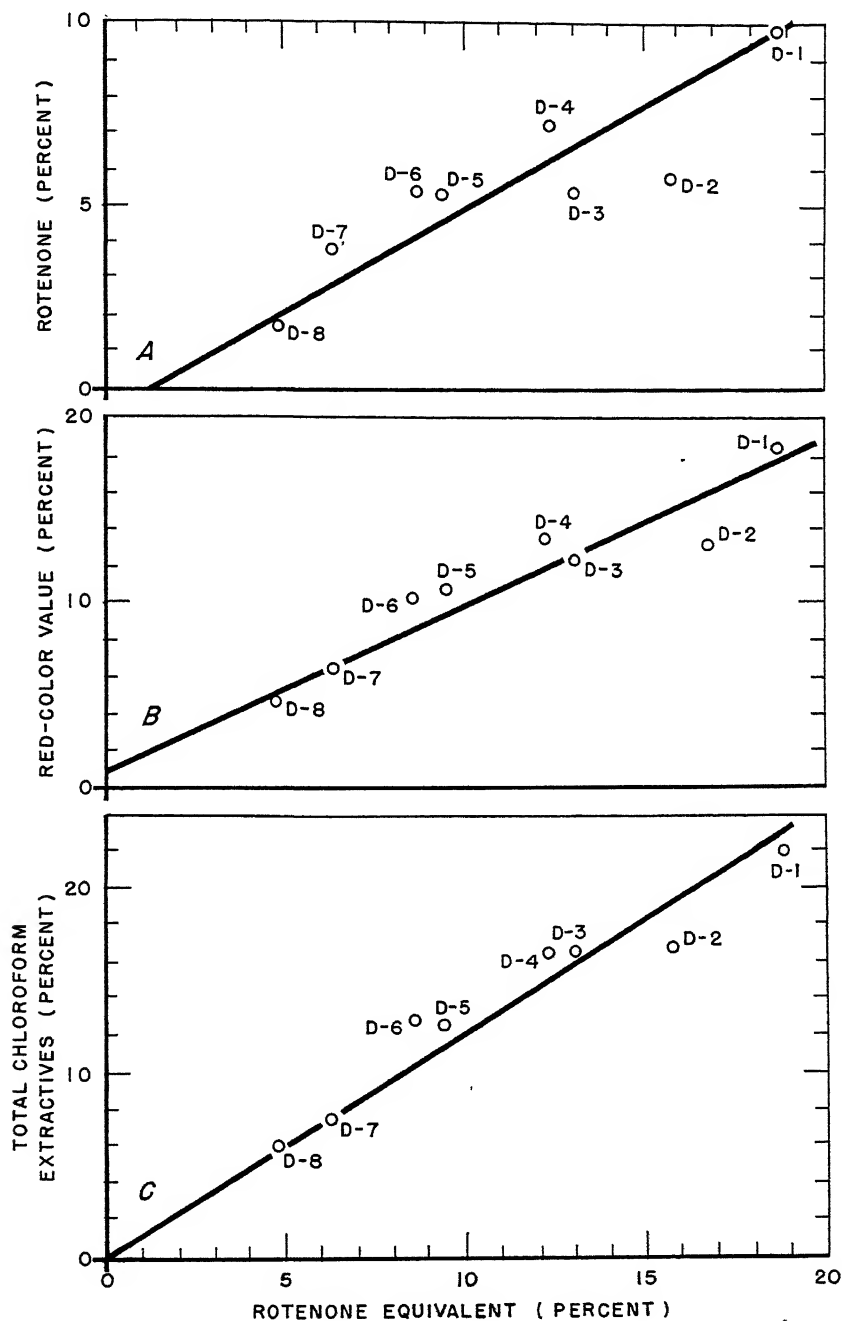
FIGURE 1.—Plot of regression lines between rotenone equivalent and rotenone content (*A*), red-color value (*B*), and total chloroform extractives (*C*) of eight derris samples.

sort has been reported for derris, but Acree et al. (*1*) found that *Amorpha fruticosa* L. gave a red color but did not contain rotenone. Likewise, Norton and Hansberry (*7*) isolated a nontoxic rotenoidlike compound from yam beans (*Pachyrhizus erosus* (L.) Urban) which gave a positive reaction to the Goodhue red-color test. Apparently, there may be substances in some roots that give the red-color test but have no toxicological value. When the regression line obtained with total chloroform extractives was projected (fig. 1, *C*), the line intersected the origin. This fact furnishes further evidence that total chloroform extractives are a better criterion of toxicity than red-color value or rotenone. This finding is in agreement with that of Ebeling et al. (*3*), who showed that the complete extractives were a more effective insecticide than equal concentrations of either the rotenone or the rotenone-free extractives used by themselves.

Table 4 shows a comparison between the toxicological rotenone equivalent of some derris samples and the values obtained from the regression equation of each of the chemical criteria. It is evident from this table that rotenone is the least accurate criterion of toxicity. The values for rotenone varied as much as 65 percent from the rotenone equivalent as determined biologically. Total chloroform extractives and red-color value were more closely in agreement with the toxicological value. The former varied as much as 17 percent and the latter up to 13 percent from the rotenone equivalent. If these deviations are compared with the error of the biological method, which in some cases was as high as 9 percent, they cannot be considered excessive.

There was no statistically significant correlation or regression between the rotenone equivalent and any of the constituents studied in lonchocarpus roots. The correlation and regression between rotenone and rotenone equivalent was almost negatively significant with a value of $r = -0.832$. For significance at the 5-percent level the correlation coefficient should be 0.878.

Although there was no significant regression between toxicological rotenone equivalent and any of the chemical values, regression equations were calculated and regression lines plotted. It can be seen from figure 2 that total chloroform extractives and red-color value do not fit the line closely. However, rotenone is in good agreement with the regression line. This indicates that rotenone, although it correlates negatively, is the best indication for toxicity in lonchocarpus roots. But in view of the small number of samples used this conclusion can only be regarded as tentative.

In table 5 are presented for comparison the toxicological rotenone equivalent of the lonchocarpus samples and the values calculated from the regression equation of each of the chemical criteria. The least deviation from rotenone equivalent is found in the values calculated from rotenone (11 percent) followed by those from total chloroform extractives (13 percent) and red-color value (14 percent).
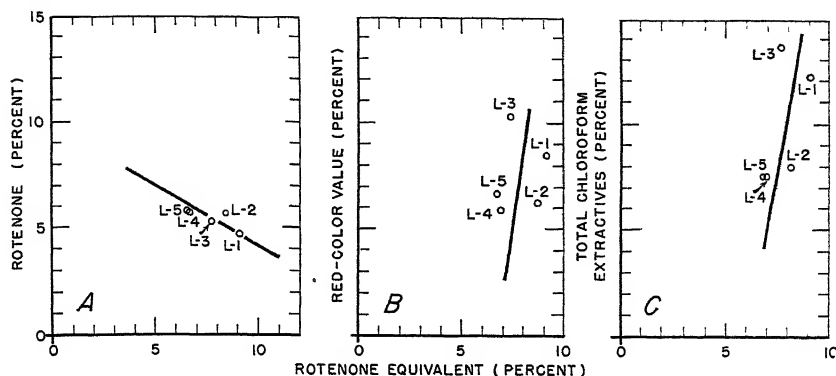
FIGURE 2.—Plot of regression lines between rotenone equivalent and rotenone content (*A*), red-color value (*B*), and total chloroform extractives (*C*) of five lonchocarpus samples.

TABLE 5.—*Comparison of biological rotenone equivalent of 5 lonchocarpus samples with that calculated from rotenone content, red-color value, and total chloroform extractives*

Sample No.	Variety	Rotenone equivalent [1]	Rotenone		Red-color value		Total chloroform extractives	
			Actual	Calculated [2]	Actual	Calculated [2]	Actual	Calculated [2]
		Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
L-1	*L. utilis*	9 0	4 7	9. 1	8 8	7. 9	12. 4	8. 1
L-2	....do	8. 5	5 5	7 6	6. 4	7 5	8 3	7. 5
L-3	.....do	7. 7	5 4	7. 8	10 6	8. 2	14 0	8 4
L-4	*L. chrysophyllus*	6 8	5 7	7. 2	6. 2	7. 5	7. 7	7 4
L-5	.....do	6. 7	5. 8	7. 0	7. 0	7. 6	7. 8	7. 4

[1] Determined biologically on houseflies
[2] These values are rotenone equivalents calculated from the respective regression equations.

DISCUSSION AND CONCLUSIONS

Insofar as the results presented here are applicable to field conditions in general, it is evident that rotenone content is not an accurate indication of the toxicological value of derris roots. It is a well-established fact that rotenone is an efficient and versatile insecticide, but it is also apparent that it is only one of several constituents in derris roots which are capable of killing insects. The best criterion of toxicity would appear to be one which would measure not only rotenone but the other toxic constituents as well. The red-color value seems to correlate more closely with rotenone equivalent than rotenone content alone because it includes such rotenoid type compounds as deguelin. However, since there may be other toxic substances in the root which do not give the red-color test, this criterion in some instances may not indicate the total toxicological value of the roots. The red-color value has the added disadvantage, as pointed out previously, of requiring special equipment and also rather exacting conditions. On the other hand, total chloroform extractives are very

simple to determine and require a minimum of time and equipment. In addition to this, the total chloroform extractives appear to be the most accurate criterion of toxicity of derris roots. This apparently is because all, or almost all, of the insecticidal constituents of derris roots are soluble in chloroform and, therefore, are readily extracted by this solvent. Since waxes, resin, and other plant material may dissolve in chloroform it is not surprising that the value for total chloroform extractives as such is usually somewhat higher than that of the rotenone equivalent as determined biologically. However, from the correlation coefficient obtained it is apparent that the ratio of the nontoxic constituent of the derris root to the toxic constituents is fairly constant and thus the total chlorform extractives multiplied by the appropriate factor give a good indication of the insecticidal value of the roots. The factor calculated from the eight available derris samples was 0.78.

In the case of lonchocarpus, neither the total chloroform extractives nor the red-color value appears to be a very good criterion of toxicity, whereas rotenone content, although it correlates negatively with toxicological value, seems to be more indicative. However, since the number of samples available for comparison is relatively small, this may not always be the case.

Although both *Lonchocarpus* and *Derris* contain rotenone, they are two distinct genera and apparently have different toxicological effects on insects. The data presented in this paper strongly suggest that they should be evaluated and treated separately.

SUMMARY

The data presented furnish additional evidence that rotenone content is not an accurate indication of the toxicological value of derris roots.

The red-color and the transmittance values give a good indication of the toxicity of derris root, but they have the disadvantage of requiring a spectrophotometer and special technique, and thus are not suitable for rapid assays.

The percentage of total chloroform extractives appears to be the simplest and most accurate criterion for estimating the toxicity of derris roots. In the eight derris samples tested the toxicity (rotenone equivalent) was 0.78 of the value for total chloroform extractives.

In the case of lonchocarpus root, neither the total chloroform extractives nor the red-color or transmittance values appear to be very good criteria of toxicity. The rotenone content, although negatively correlated with toxicological value, seems to be more indicative.

The results of these laboratory tests and of those from practical field trials strongly suggest that derris and lonchocarpus roots, although they both contain rotenone, should be evaluated separately.

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TRISTEZA DISEASE OF CITRUS [1]

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INTRODUCTION

Tristeza, a serious citrus disease that occurs in all the major citrus-growing districts of Brazil, Argentina, and Uruguay, now seems similar to or identical with serious disorders of sweet orange (*Citrus sinensis* (L.) Osbeck) on sour orange (*C. aurantium* L.) rootstock reported from the Union of South Africa, Java, Australia, and southern California. Methods of transmitting this disease and the susceptibility of various species and varieties of citrus were studied cooperatively in Campinas, Brazil, from 1946 to 1948, symptoms of the disease were observed, and the methods of control tried in Brazil were evaluated. The results of these studies, preliminarily reported in 1947 (*1*), [3] are given herein, together with the history of ,the disease, the causes assigned to it, and a discussion of scion-stock relation to the disease and the possibility of virus strains.

HISTORY AND CAUSE OF TRISTEZA

The effects of the disease now known as tristeza in Brazil and as podredumbre de las raicillas in Argentina and Uruguay probably have been known for more than half a century. However, only after the appearance of the disease in the citrus-producing areas of Argentina and Brazil, where it caused heavy losses to the citrus industries,

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was it recognized as a distinct disease. Not until its potentialities for causing destruction were fully appreciated were extensive efforts made to determine its nature and control.

The disease appears to have originated in the Union of South Africa, supposedly in some plant native to that country. Early in the development of the citrus industry there, it was found that varieties of sweet orange did not thrive when grafted to sour orange and soon sour orange was abandoned as a rootstock (*35*). Later, efforts to reintroduce sour orange as a rootstock met with failure. Trees of the scion-stock combinations adopted for general use, largely sweet orange on rough lemon (*Citrus limon* (L.) Burn. f.), showed no injury, although there is now reason to believe that the causal agent of the disease persisted in the citrus groves and that infected material was carried to other parts of the world, thus establishing new centers of the disease.

A disease similar to the South African trouble was noted in Java in 1928, when efforts were made to use sour orange as a rootstock for varieties of sweet orange (*33*).

Tristeza was discovered in Argentina in 1930 or 1931 (*9, 10*). It was found in the Paraíba Valley of Brazil about 1937 (*4*) and was observed in Uruguay in 1940 (*3*).

In 1939 a similar disease was found in a citrus grove in California (*12*). Apparently on account of the rapid decline of affected trees in the first plantings in which it was observed, this disease was called quick decline.

A disease, designated as bud-union decline, which caused decline and death principally of trees of sweet orange and grapefruit on sour orange rootstock was observed in Victoria, Australia, in 1941 (*16*). By 1947 it had caused considerable damage to the citrus industry of that State.

For many years the failure of trees of sweet orange on rootstock of sour orange to thrive in the Union of South Africa was considered to be due to a type of incompatibility between stock and scion. The same condition or a similar one discovered in Java was considered by Toxopeus (*33*) to be due to a type of scion-stock incompatibility in which the sweet orange scion produced a substance toxic to the sour orange stock. After tristeza was discovered in South America, many theories were put forth to explain the effects produced. These include scion-stock incompatibility, soil acidity, various types of nutrient deficiencies, soil toxins, high soil-moisture content, prolonged dry periods, nematode infestations, pathogenic organisms, and infection by a virus.

Bitancourt (*5*) stated that, as a result of observations made on podredumbre de las raicillas in the Province of Corrientes, Argentina, in 1937, he and Fawcett had suggested that one of the possible causes of the disease might be a virus latent in the sour orange and injurious to the sweet orange top. In the same paper Bitancourt explained that the most acceptable hypothesis as to the cause of the disease is that an infectious agent is involved. In 1943 Webber (*35*) proposed the hypothesis that the disease is caused by a virus latent in sweet orange but lethal to sour orange and that regularly and normally the foliage of sour orange and lemon produces some product that inhibits

the action of the virus. In 1944 Bitancourt (7) presented the theory that the disease is caused by a virus which is latent in the sweet orange and from which the sour orange is completely immune.

The first experimental evidence supporting the hypothesis that tristeza is caused by a virus was obtained by Meneghini (18), who reported in 1946 that the disease is transmitted by an aphid. The vector was identified tentatively as *Aphis tavaresi* Del Guercio; but probably it is more properly called *A. citricidus* (Kirk.), for which *A. tavaresi* and *A. citricola* Van der Goot appear to be synonyms. In 1947 Bennett and Costa (1) reported the confirmation of Meneghini's transmission results with *A. citricidus*. They reported also transmission of the disease in greenhouse and field tests in which buds and twigs from diseased trees were grafted to sweet orange plants.

Fawcett and Wallace (13) in 1946 and Wallace and Fawcett (34) in 1947 reported transmission of quick decline in California by buds from diseased trees, and Oberholzer (21) in 1947 reported results indicating bud transmission of the disease that causes failure of trees of sweet orange on sour orange rootstock in the Union of South Africa.

It now seems well established that tristeza in Brazil is caused by a virus that is transmitted by the oriental citrus aphid (*Aphis citricidus*). At certain seasons this aphid is found in abundance on citrus trees in southern Brazil. It occurs also in Argentina and Uruguay; and, according to Takahashi (31, p. 47), it has been reported from Japan, China, India, Java, Sumatra, Ceylon, Hawaiian Islands, South America, and Africa. Apparently it has not been found in the United States.

ECONOMIC IMPORTANCE AND SPREAD OF TRISTEZA IN INVADED AREAS

The spread of tristeza in South America, as described by several investigators (2, 7, 8, 20), has been relatively rapid. The disease probably now occurs in all the major citrus-producing areas of Brazil, Argentina, and Uruguay. In about 18 years it has killed or rendered of little commercial value a high percentage of the orange trees on sour orange rootstock in these countries.

In the State of São Paulo, Brazil, the disease spread in about 12 years to all the citrus-producing areas, infecting and destroying upward of 6,000,000 trees, or about 75 percent of the orange trees of the State.

The spread of the disease within groves also has been rapid after it became established. For more than 6 years Moreira [4] followed the spread of tristeza in a grove of 2,606 trees of the variety Bahianinha on sour orange rootstock, located at the Estação Experimental de Citricultura de Limeira, and obtained the following results:

Date of observation:	Trees affected with tristeza	
	Number	*Percent*
November 28, 1942	308	11.8
October 8, 1943	508	19.5
January 4, 1945	1,435	55.1
January 10, 1946	1,890	72.5
January 19, 1948	2,606	100.0

[4] Unpublished information made available to A. S. Costa by Sílvio Moreira.

In most groves of sweet orange on sour orange rootstock affected with tristeza large numbers of trees have died after a few years. However, in many instances factors other than tristeza probably are important in the short life of the affected trees. Diseased groves soon become unprofitable and are abandoned. The diseased trees then suffer from weed competition and general lack of care, which contribute materially to the rate of decline. This is indicated by the observations of Moreira in the grove at Limeira already mentioned, where, after more than 6 years during which the trees had good care, only 15.5 percent of the trees had died; at the end of the period, however, none of the trees had commercial value.

SYMPTOMS OF TRISTEZA

The first evidences of tristeza in older orange trees usually are a partial to almost complete suppression of new flushes of growth and the production of various types of leaf discolorations. The first symptoms may appear on only one branch, but often a greater part of the tree is involved. Older leaves become dull or slightly bronzed at first; later they may take on various shades of yellow, the amount of yellowing apparently depending on environmental conditions and on the variety involved. The Barão variety of sweet orange appears to be more subject to leaf yellowing than some other varieties. Yellowing may occur uniformly over an entire leaf, or it may be more conspicuous in some parts than in others; often it is more intense in the midrib or in the midrib and lateral veins. Occasionally diseased leaves tend to stand upright. Diseased trees with yellow leaves are conspicuous for considerable distances.

Soon after leaf discoloration begins, the older leaves at the base of the twigs begin to fall. Usually leaf fall continues progressively toward the tip until many of the twigs are defoliated or have only a few of the younger leaves remaining. In many cases abscission takes place between the petiole and the leaf blade, the petiole remaining attached to the twig for a time after the blade has dropped. As leaf fall continues, the limbs of the tree become exposed. Weak shoots begin to grow out of axillary buds, mostly in a vertical position; these shoots produce small leaves, paler than normal ones and sometimes with yellow midribs. Limbs begin to die back from the tips, and more weak shoots are produced from the main limbs and trunk. After a tree has shown symptoms for 2 to 3 years, in most cases nearly all the smaller limbs are dead and the tree is devoid of foliage except for a few weak shoots on the main limbs and trunk. Under average conditions twig growth usually becomes weaker each season until the tree dies. Some trees look, however, as if they might remain alive indefinitely, with a reduced number of weak shoots.

Decline, however, does not follow this general pattern in all cases. Some trees collapse quickly, but such collapse appears to be relatively rare. Others decline slowly and finally reach a stage at which they produce sparse foliage, more or less normal in color, relatively few blossoms, and light crops of fruit of inferior quality. Some affected trees seem capable of persisting indefinitely in this state of reduced vigor.

Diseased trees have a tendency to blossom heavily in the earlier stages of attack and usually set heavy crops of fruit. When such fruits begin to take on color, they are more conspicuous than those on healthy trees, partly because of the smaller amount of foliage on the diseased trees.

Trees with symptoms of tristeza in the tops also show severe root injury. There is a marked depletion of starch in the earlier stages in the rootlets and later in the larger roots. First the rootlets die and decay, and later the injury extends to the larger roots until the capacity of the tree to take up water and mineral nutrients is greatly impaired. In general, top symptoms on larger trees are those that may be produced as a result of root injury.

Bitancourt (7) found also that in diseased trees there is evidence of a greater concentration of starch in the outer bark of the sweet orange scion immediately above the graft union than in the outer bark of the sour orange stock. Rossetti (25) found this condition to prevail in most of the healthy-appearing trees as well as in obviously diseased ones of susceptible scion-stock combinations at the Estação Experimental de Citricultura de Limeira. It has not been determined, however, that starch concentration in the bark can be used to detect infection well in advance of the production of symptoms in the foliage.

Starch depletion from the roots and accumulation in parts of the tops of trees appear to be associated with necrosis in the phloem, shown by Schneider, Bitancourt, and Rossetti (29) to occur in the bark of diseased trees in the region of the bud union. A similar type of necrosis in trees affected with quick decline was described by Schneider (27).

It has not been feasible as yet to determine the incubation period of the disease on larger trees. On young trees in nursery rows symptoms

FIGURE 1.—Orange variety Bahianinha on sour orange rootstock photographed 10 months after inoculation: *A*, Noninoculated (check) trees (first five in row); *B*, trees inoculated by insertion of buds from diseased sweet orange trees (first five in row).

In most groves of sweet orange on sour orange rootstock affected with tristeza large numbers of trees have died after a few years.   However, in many instances factors other than tristeza probably are important in the short life of the affected trees.   Diseased groves soon become unprofitable and are abandoned.   The diseased trees then suffer from weed competition and general lack of care, which contribute materially to the rate of decline.   This is indicated by the observations of Moreira in the grove at Limeira already mentioned, where, after more than 6 years during which the trees had good care, only 15.5 percent of the trees had died; at the end of the period, however, none of the trees had commercial value.

SYMPTOMS OF TRISTEZA

The first evidences of tristeza in older orange trees usually are a partial to almost complete suppression of new flushes of growth and the production of various types of leaf discolorations.   The first symptoms may appear on only one branch, but often a greater part of the tree is involved.   Older leaves become dull or slightly bronzed at first; later they may take on various shades of yellow, the amount of yellowing apparently depending on environmental conditions and on the variety involved.   The Barão variety of sweet orange appears to be more subject to leaf yellowing than some other varieties.   Yellowing may occur uniformly over an entire leaf, or it may be more conspicuous in some parts than in others; often it is more intense in the midrib or in the midrib and lateral veins.   Occasionally diseased leaves tend to stand upright.   Diseased trees with yellow leaves are conspicuous for considerable distances.

Soon after leaf discoloration begins, the older leaves at the base of the twigs begin to fall.   Usually leaf fall continues progressively toward the tip until many of the twigs are defoliated or have only a few of the younger leaves remaining.   In many cases abscission takes place between the petiole and the leaf blade, the petiole remaining attached to the twig for a time after the blade has dropped.   As leaf fall continues, the limbs of the tree become exposed.   Weak shoots begin to grow out of axillary buds, mostly in a vertical position; these shoots produce small leaves, paler than normal ones and sometimes with yellow midribs.   Limbs begin to die back from the tips, and more weak shoots are produced from the main limbs and trunk. After a tree has shown symptoms for 2 to 3 years, in most cases nearly all the smaller limbs are dead and the tree is devoid of foliage except for a few weak shoots on the main limbs and trunk.   Under average conditions twig growth usually becomes weaker each season until the tree dies.   Some trees look, however, as if they might remain alive indefinitely, with a reduced number of weak shoots.

Decline, however, does not follow this general pattern in all cases. Some trees collapse quickly, but such collapse appears to be relatively rare.   Others decline slowly and finally reach a stage at which they produce sparse foliage, more or less normal in color, relatively few blossoms, and light crops of fruit of inferior quality.   Some affected trees seem capable of persisting indefinitely in this state of reduced vigor.

Diseased trees have a tendency to blossom heavily in the earlier stages of attack and usually set heavy crops of fruit. When such fruits begin to take on color, they are more conspicuous than those on healthy trees, partly because of the smaller amount of foliage on the diseased trees.

Trees with symptoms of tristeza in the tops also show severe root injury. There is a marked depletion of starch in the earlier stages in the rootlets and later in the larger roots. First the rootlets die and decay, and later the injury extends to the larger roots until the capacity of the tree to take up water and mineral nutrients is greatly impaired. In general, top symptoms on larger trees are those that may be produced as a result of root injury.

Bitancourt (7) found also that in diseased trees there is evidence of a greater concentration of starch in the outer bark of the sweet orange scion immediately above the graft union than in the outer bark of the sour orange stock. Rossetti (25) found this condition to prevail in most of the healthy-appearing trees as well as in obviously diseased ones of susceptible scion-stock combinations at the Estação Experimental de Citricultura de Limeira. It has not been determined, however, that starch concentration in the bark can be used to detect infection well in advance of the production of symptoms in the foliage.

Starch depletion from the roots and accumulation in parts of the tops of trees appear to be associated with necrosis in the phloem, shown by Schneider, Bitancourt, and Rossetti (29) to occur in the bark of diseased trees in the region of the bud union. A similar type of necrosis in trees affected with quick decline was described by Schneider (27).

It has not been feasible as yet to determine the incubation period of the disease on larger trees. On young trees in nursery rows symptoms

Figure 1.—Orange variety Bahianinha on sour orange rootstock photographed 10 months after inoculation: *A*, Noninoculated (check) trees (first five in row); *B*, trees inoculated by insertion of buds from diseased sweet orange trees (first five in row).

have been observed occurring naturally 18 months after budding. Trees 1 to 2 years old may produce symptoms 4 months after inoculation, and small plants in pots produce yellow leaves in periods as short as 30 days. Symptoms in small trees vary greatly, but usually affected plants are somewhat unthrifty in appearance and the leaves range in color from slightly more dull than normal to yellow. Diseased trees may blossom and set a crop of fruit the first year after being transplanted from the nursery (fig. 1). Symptoms on young trees are described in greater detail on pages 213, 217, and 219.

TRANSMISSION OF TRISTEZA

Attempts were made to transmit tristeza of citrus to nursery-grown orange trees, to small potted sweet orange trees on sour orange rootstock in the greenhouse, and to various other species of plants, mostly annuals, that were readily available. Methods of transfer included grafting and budding from diseased to healthy orange trees, inoculation with juice from diseased orange trees, training dodder from diseased to healthy plants, and infesting plants with various species of insects found to feed on affected trees.

INOCULATION OF NURSERY-GROWN TREES BY BUDDING

In the first test of transmission of tristeza to trees in the field, 20 trees of Bahianinha sweet orange on sour orange rootstock were selected from a nursery near Limeira and planted in plots at Campinas on October 31, 1946. Ten of these plants were inoculated on November 20 by inserting 2 buds from diseased trees in each. The trees were reinoculated on December 18. The remaining 10 trees were held as checks. Because these trees came from an area where tristeza was prevalent, some of them may have been infected naturally before inoculation.

One of the check trees began to produce yellow leaves in mid-January and showed other symptoms characteristic of tristeza. By June 1, 1947, the 10 inoculated trees and 3 of the check trees showed rather marked symptoms of tristeza. All the diseased trees blossomed, and 9 of the 10 inoculated trees set fruit which matured in early September (fig. 1, *B*). All the inoculated trees and the 3 diseased check trees blossomed heavily again in August and early September. By October 1 the 7 healthy-appearing check trees were considerably larger than the inoculated trees, had normal green foliage, had blossomed sparsely, and had set no fruit. By March 30, 1948, 1 of the 10 inoculated trees had died; 9 were markedly stunted in comparison with the check trees, but 8 of these showed some degree of recovery in the leaves. Of the 10 check plants, 4 showed some stunting without chlorosis and 1 of the larger trees was beginning to produce chlorotic leaves in which vein yellowing was evident.

In a second test 5 to 25 trees of each of 11 varieties of sweet orange budded on sour orange rootstock which had been obtained from a nursery near Santa Rita during the early part of December 1946 were planted in plots at Campinas (table 1). At the time the trees were selected tristeza was beginning to appear in some of the commercial groves at Santa Rita, but since the majority of the citrus trees appeared

healthy it was hoped that most of the young trees selected would prove to be free of infection. At least half of the trees of each variety were inoculated by insertion of 2 buds from diseased trees into each tree on January 18, 1947, and were reinoculated with the same number of buds on April 9. There was a high mortality in the buds of the first inoculation, but those of the second survived reasonably well. No buds were inserted into the check trees.

TABLE 1.—*Results of inoculating nursery trees of sweet orange on sour orange rootstock by means of buds from diseased trees, Campinas, Brazil, 1946–47*

[Test 1· Trees planted Oct. 31, 1946, inoculated Nov. 20 by insertion of 2 buds from diseased trees into each tree, and reinoculated Dec. 18 with buds from same source. Test 2: Trees planted during first week of December 1946, inoculated Jan. 18, 1947, and reinoculated Apr. 9. Results recorded Oct. 1, 1947]

Test and variety [1]	Trees in-oculated	Inoculated trees diseased	Check trees	Check trees diseased
Test 1·	*Number*	*Number*	*Number*	*Number*
Bahianinha	10	10	10	3
Test 2:				
Abacaxí	5	0	5	0
Bahianinha	5	5	0	----
Barão	15	10	10	0
Campista	5	5	5	0
Coronel	5	5	5	1
Lima	5	4	5	0
Mangaratiba	5	3	5	0
Parnaso	5	5	5	0
Pera do Rio	10	9	10	0
Seleta	15	13	10	0
Serrana	5	4	5	0

[1] Varieties are listed by the names under which they were received from the nursery. These trees were inspected in early 1948 by Sílvio Moreira, and it is his opinion that the varieties labeled Coronel and Lima are Laranja Serra Dagua and Serrana de Limeira, respectively, and that the variety labeled Campista is Laranja Coco.

By the first of May, or about $3\frac{1}{2}$ months after the first inoculation, some of the inoculated trees of the variety Barão began to show an abnormal coloration of older leaves, consisting chiefly of a dull appearance with a suggestion of vein yellowing. About a month later some of the inoculated trees of the variety Seleta began to show abnormal coloration of the foliage. Symptoms considered to be caused by tristeza appeared in other varieties, and by October 1 most of the inoculated trees of all of the varieties except Abacaxí appeared to be diseased (table 1). Abnormalities consisted chiefly of a certain degree of dullness of the older leaves and of various types and degrees of yellowing of the foliage including some vein yellowing. As symptoms increased in severity the plants became slightly stunted, new growth was partially suppressed, and many of the diseased trees blossomed heavily.

Leaf cast occurred to some extent on some of the trees of the varieties Barão and Mangaratiba, and it was especially heavy on all the inoculated trees of the variety Coronel (fig. 2). There was considerable variation among the varieties in the degree and type of yellowing of the foliage. Vein yellowing was more evident in mature leaves of the variety Barão than in those of other varieties. Some vein yellowing was evident also in the leaves of the Mangaratiba, but the most outstanding characteristic on this variety was the rather uniform yellowing of the leaves on some of the younger shoots. Leaf dropping was followed by the production of short, weak shoots along the main
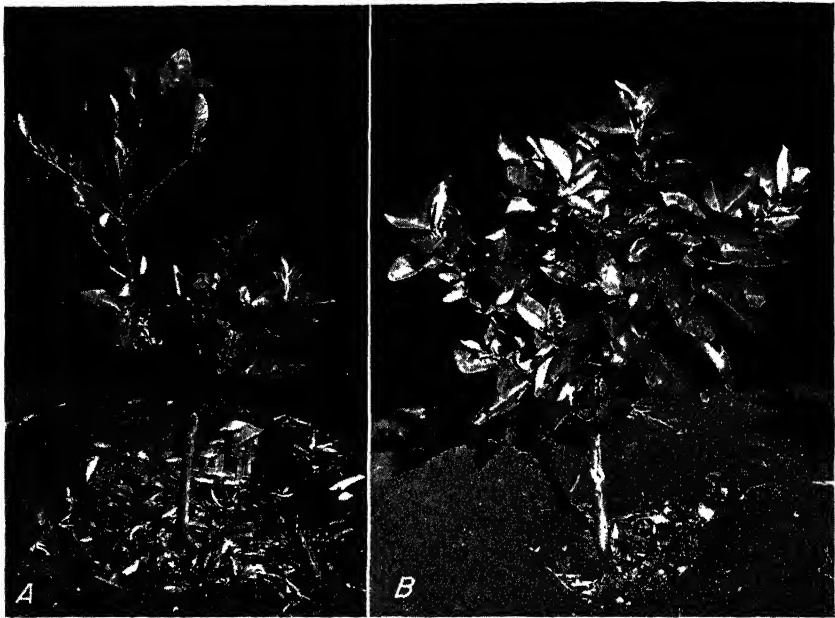
FIGURE 2.—Young orange trees of the variety Coronel on sour orange rootstock planted in field at Campinas, Brazil, during the early part of December 1946 and photographed about 7 months after inoculation: *A*, Tree inoculated by insertion of buds from diseased sweet orange trees on January 18, 1947; *B*, noninoculated (check) tree.

limbs. Except for one tree of Coronel, which lost many of its leaves, blossomed heavily, and was obviously diseased, none of the check trees showed symptoms characteristic of tristeza at the time of these observations.

Tests were made at intervals on inoculated and noninoculated groups of plants of this experiment by Bitancourt's method (7) to determine the relative amounts of starch in the outer bark of the sweet and the sour orange in the vicinity of the graft union. In some cases results of the starch test correlated reasonably well with the conditions of the tops, but in most cases they were erratic. By the method suggested by Fawcett (11) for quick decline, tests were made to determine starch content of the roots of all the trees of this experiment about 6 months after inoculation and about 2 months after some of the trees had begun to show symptoms of the disease in the tops. No marked evidence of starch depletion was observed in the roots of inoculated plants at that time; however, it is presumed that starch depletion will take place in these plants as the disease progresses.

Iodine tests made about 6 months after inoculation indicated that leaves of inoculated plants had more starch than those of noninoculated plants. Starch determination [5] showed that in two samples of Bahianinha leaves the starch content averaged 0.742 and 0.346 percent, respectively, for inoculated and noninoculated trees and in three

[5] Made by José Pio Nery.

samples of Seleta leaves, 1.192 and 0.607 percent, respectively, for inoculated and noninoculated trees. One determination gave 0.920 and 0.373 percent starch, respectively, in leaves of girdled and non-girdled limbs of a healthy Bahianinha tree, indicating that starch accumulation in the leaves of girdled healthy twigs may be of about the same order as that in diseased orange trees in certain stages of disease. Discoloration of the leaves of the girdled branches was more or less similar to that of leaves of some of the inoculated trees in this test.

INOCULATION OF GREENHOUSE PLANTS

An extensive series of inoculation tests was made in greenhouses screened to give a considerable degree of protection against insects. Most of the inoculations were made on small, rapidly growing, potted plants of sweet orange on sour orange rootstock. The plants used in these tests were obtained by grafting sweet orange seedlings on sour orange seedlings, by a method already described (*1*), when the respective seedlings were 6 to 10 cm. tall.

JUICE INOCULATIONS

Succulent twigs from badly diseased trees of sweet orange on sour orange rootstocks were selected to serve as sources of inoculum for juice inoculations. The twigs were ground in a meat chopper, and the sap was extracted from the macerated leaf and twig material with a small press and used immediately. Inoculations were made by first sprinkling the plants with an abrasive and then rubbing the leaves with a cloth pad saturated with inoculum.

In 1 experiment 40 vigorously growing orange plants about 15 cm. tall were inoculated. None of these plants showed symptoms of tristeza after 4 months. The 40 check plants also remained healthy. In a second experiment several methods of mechanical inoculation were employed in inoculating 50 small plants of the variety Barão on sour orange rootstock, but none of these and none of the 50 check plants of this test showed evidence of infection.

In addition, 67 species and varieties of plants of various types were inoculated in an effort to find a plant susceptible to infection by juice inoculation. Most of these species were annuals grown in pots from seeds. Twenty-five or more plants of most kinds were inoculated when relatively small and growing rapidly. No symptoms considered to be caused by the virus of tristeza were observed on any of these plants.

TESTS FOR DODDER TRANSMISSION

During the past few years several viruses have been transmitted by dodder (*Cuscuta* spp.). This method of transmission has been particularly successful with viruses that cause yellowing or leaf curling and that appear to have a close association with the phloem of the host plant. Evidence of phloem collapse presented by Schneider, Bitancourt, and Rossetti (*29*) suggests that tristeza may be caused by a virus of this type, but this evidence alone is not sufficient to justify final conclusions.

Five species of dodder (*Cuscuta americana* L., *C. campestris* Yuncker, *C. indecora* Choisy, *C. subinclusa* Durand and Hilgard, and *Cuscuta* sp. collected near Campinas) were used in transmission tests. All these except *C. campestris* grew well on orange trees. In the inoculation tests the dodder was first established on succulent shoots of diseased orange trees growing in barrels in the greenhouse. At intervals over a period of about 7 months stems of dodder were trained from the diseased trees to healthy plants of various kinds. After about 7 days the stems connecting the diseased trees with the inoculated plants were broken. The inoculated plants were kept under observation for several months. The species and varieties of plants inoculated and the results obtained are presented in table 2.

TABLE 2.—*Results of attempts to transmit tristeza virus by training dodder from infected orange trees and allowing it to become established on healthy plants of various kinds*

Species of dodder (*Cuscuta*) tested	Plant inoculated [1]	Plants inoculated	Plants infected
		Number	*Number*
C. americana	Citrus sinensis (sweet orange)	20	0
	Daucus carota L. (carrot)	13	0
	Datura stramonium L. (jimsonweed)	12	0
	Euphorbia prunifolia Jacq (amendoim bravo)	6	0
	Lycopersicon esculentum Mill. (tomato)	10	0
	Nicotiana glauca Graham (tree tobacco)	4	0
	Nicotiana glutinosa L	5	0
	Nicotiana tabacum L. (tobacco, var. Turkish)	10	0
	Phytolacca americana L. (pokeweed)	14	0
	Sida rhombifolia L	5	0
C. campestris	Citrus sinensis (sweet orange)	11	0
C. indecora	----do----	20	0
	Lycopersicon esculentum (tomato)	16	0
	Nicotiana glauca (tree tobacco)	6	0
	Phytolacca americana (pokeweed)	8	0
C. subinclusa	Vinca rosea L. (periwinkle)	15	0
	Citrus sinensis (sweet orange)	20	1
Cuscuta sp	Beta patellaris Moq	19	0
	Citrus sinensis (sweet orange)	20	1 (?)
	Daucus carota (carrot)	14	0
	Lycopersicon esculentum (tomato)	20	0
	Nicotiana glutinosa	11	0
	Nicotiana tabacum (tobacco, var. Turkish)	9	0
	Phytolacca americana (pokeweed)	12	0
	Physalis sp (S-51)	20	0
	Physalis sp. (S-92)	6	0
	Vinca rosea (periwinkle)	6	0

[1] All plants of *Citrus sinensis* were on sour orange rootstock.

None of the noncitrus plants used in these tests showed evidence of disease. However, one of the sweet orange plants on which *Cuscuta subinclusa* was established developed yellowing and stunting typical of plants affected with tristeza. Virus was transmitted from this plant to other orange plants by means of *Aphis citricidus*. Also one sweet orange plant on which *Cuscuta* sp. was established began to produce yellow leaves after about 2 months. Yellowing was still evident after 3 months and the plant was stunted, but no attempts were made to recover tristeza virus. Of 20 check plants of sweet orange on which dodder (*Cuscuta subinclusa*, 10 plants; *Cuscuta* sp., 10 plants) from healthy orange plants was established, none showed symptoms of tristeza after 6 months.

Further tests with larger numbers of plants are needed before definite conclusions may be drawn regarding transmissibility of the tristeza virus by dodder. However, the available evidence indicates

that the virus may be transmissible to a low percentage of sweet orange plants by certain species. Transmission to a low percentage of plants or to no plants would indicate that dodder is not a host of the tristeza virus.

<div align="center">TRANSMISSION BY TISSUE UNION</div>

Attempts were made to transmit tristeza to small potted plants of sweet orange on sour orange rootstock by means of buds and twig grafts. In the attempts to transmit the disease by budding, vigorously growing plants 30 to 60 cm. tall were inoculated by inserting one small bud from a diseased plant in the sweet orange scion of each healthy plant a short distance above the graft union with the sour orange rootstock. Of 40 plants inoculated with buds in this manner, 15 later showed symptoms characteristic of tristeza; whereas, 40 plants of the same lot not budded remained healthy.

Two methods of inoculation by grafting twigs from diseased plants to healthy ones were used. In the first method potted sweet orange plants 30 to 50 cm. tall were placed adjacent to diseased plants growing in pots or barrels. A downward incision was made in the stem of the plant to be inoculated, and a similar but inverted incision was made in an adjacent twig of the diseased plant. The V-shaped segment of stem of the diseased twig was fitted into the incision of the healthy plant, and the two twigs were bound together firmly with a cord. After about 30 days each diseased twig was severed just below the new graft union. Of 26 plants inoculated in this manner, 21 showed definite symptoms of tristeza. Of the 10 check trees grafted with twigs from healthy plants, none showed evidence of infection.

In the second method of inoculation suitable twigs were taken from diseased trees and grafted to the stems of potted plants by the method just described. The lower ends of the twigs were kept in vials of water for 30 days or more. After union was complete the stems were severed just below the new graft union. In three separate tests this method of inoculation resulted in high percentages of infection. Of 15 plants inoculated, 14 showed symptoms of tristeza. Of 15 check plants of the same lot not grafted with twigs, all remained free of symptoms.

Plants grafted with twigs that remained attached to diseased trees for 30 days or more showed symptoms of disease in a relatively short time. Usually within 30 days after inoculation new leaves at the tips of the plants began to show a pale-yellow color. Further growth resulted in more yellow leaves, and in some cases the older leaves of the plants also became yellow; in other plants the older leaves did not turn yellow but were somewhat less glossy than those of healthy plants. Stunting was marked regardless of the degree of yellowing of the foliage. After 4 to 6 months the check plants were two or three times as tall as the inoculated ones.

Plants inoculated by budding or by grafting with detached twigs were slower in showing symptoms, and the incubation period of the disease varied from 1 to 4 months. After symptoms began to appear, however, they were similar to those produced by other methods of inoculation.

TESTS FOR INSECT TRANSMISSION

The species of insects tested for ability to transmit the virus of tristeza are listed in table 3. Of these species, *Aphis citricidus*, *A. gossypii* Glov., *Macrosiphum solanifolii* (Ashm.), and *Aleurothrixus floccosus* (Mask.) were found breeding on citrus trees under field conditions. All the other species except *Macrosiphum ambrosiae* (Thos.), *Myzus persicae* (Sulz.), and *Aphis rumicis* L. were found on orange trees at different times, but it was not determined that any of them breeds on citrus. In all the tests except those with *A. citricidus* the insects were collected and placed on diseased trees on which they were allowed to feed for periods ranging from several hours to several days. The insects were then transferred to healthy plants. Large numbers of insects were used in most of the tests.

TABLE 3.—*Results of tests in which sweet orange plants on sour orange rootstock were infested with different species of insects from diseased orange trees to determine whether they are vectors of tristeza virus* [1]

Insect tested	Source of insect	Plants infested	Plants infected
		Number	*Number*
	Diseased orange tree	20	20
	Nonviruliferous colony	20	0
	Diseased orange tree	5	4
	___do	10	8
Aphis citricidus	Nonviruliferous colony	10	0
	Diseased orange tree	38	22
	Nonviruliferous colony	20	0
	Diseased orange tree	10	7
	____do	10	10
	Nonviruliferous colony	10	0
Aphis gossypii	Diseased orange tree	15	0
Aphis rumicis	____do	13	0
Macrosiphum ambrosiae	____do	5	0
Macrosiphum solanifolii	____do	15	0
	Potato	5	0
Myzus persicae	Diseased orange tree	10	0
Aleurothrixus floccosus	____do	10	0
Agallia albidula	____do	11	0
Agalliana ensigera Oman	____do	10	0
Bucephalogonia sp	____do	10	0
Empoasca batatae Poos	____do	10	0
	Sweetpotato	5	0
Miscellaneous leafhoppers	Diseased orange tree	5	0

[1] The number of insects used to infest the test plants ranged from 15 to 50 for *Bucephalogonia* sp. and from 50 to 300 for all other species, the larger numbers being used on plants infested with the different species of aphids.

In the tests with *Aphis citricidus*, infested twigs were taken from diseased orange trees and placed directly on healthy plants. The insects crawled to the healthy plants as the diseased twigs wilted. The aphids were allowed to feed 2 to 4 days and were then killed with a nicotine spray. Check plants were infested with aphids from healthy orange trees in cages. These aphids were the progeny of individuals taken from their mothers at birth and placed on healthy seedling plants. Except as noted, 100 to 300 aphids were placed on each infested plant.

A high percentage of infection was obtained in each experiment in which *Aphis citricidus* was used, but no evidence of infection was observed on plants infested with other species of insects. Some of the plants infested with *A. citricidus* began to produce yellow leaves

at the growing tips 30 days after infestation. After these leaves began
to appear growth was much retarded and old and young leaves showed
various degrees of yellowing. The leaves of some of the diseased
plants, especially those of the variety Barão, had a tendency to stand
more nearly upright than those of healthy plants. On some plants
the leaves turned bright yellow, and in a few cases they dropped.
Yellowing of other plants was less marked, and the leaves were only
slightly paler than normal. Some of the plants of this latter type
produced new shoots with small leaves more or less normal in color.
In general, the yellow plants were smaller than those with greener
foliage. All the check plants that were infested with large numbers
of aphids from nonviruliferous colonies remained healthy and grew
rapidly. Figure 3 shows an infected yellow plant and a check plant
of one experiment about 6 months after the plants were infested.

One test was made to obtain evidence as to whether relatively
small numbers of *Aphis citricidus* can effectively transmit tristeza and
whether a period of starvation before feeding on diseased plants
increases the infectivity of the aphids. For this test, aphids were
taken from the nonviruliferous colonies and placed on wet filter paper
in moist chambers. After a starvation period of 4 to 7 hours the aphids
were removed and placed on succulent twigs from diseased sweet
orange plants on which they were allowed to feed for periods ranging
from 5 to 60 minutes before being transferred to healthy plants.
Each plant was infested with 50 aphids. As one of the controls, each
plant was infested with 50 aphids taken directly from diseased plants;
as a second control, on relation of numbers of insects per plant to
infection, each plant was infested with about 300 aphids taken directly
from diseased plants. The feeding periods and the results are shown
in table 4.

TABLE 4.—*Results of transmission tests with Aphis citricidus in which nonvirulif-
erous aphids were starved for periods of 4 to 7 hours, allowed to feed for indicated
periods on diseased sweet orange twigs, and then transferred to small plants of
sweet orange on sour orange rootstock*

Source of aphids	Periods starved aphids fed on diseased plants	Aphids per plant	Plants infested	Plants infected
	Minutes	*Number*	*Number*	*Number*
Nonviruliferous colony	0	50	5	0
Do	5	50	5	0
Do	10	50	5	0
Do	30	50	5	0
Do	60	50	5	1
Diseased orange trees	(¹)	50	5	3
Do	(¹)	300	5	5

¹ Aphids reared on diseased plants, not starved; transferred directly from diseased twigs to healthy plants.

This test is too limited to permit the drawing of final conclusions,
but the results indicate that the starvation period prior to feeding on
the diseased plants did not result in greater efficiency in transmission.
They suggest also that feeding periods of 60 minutes or more are
required for the insect to become fully charged with virus. Fifty
aphids from a diseased tree appeared less effective in transmitting the
virus than 300 aphids.
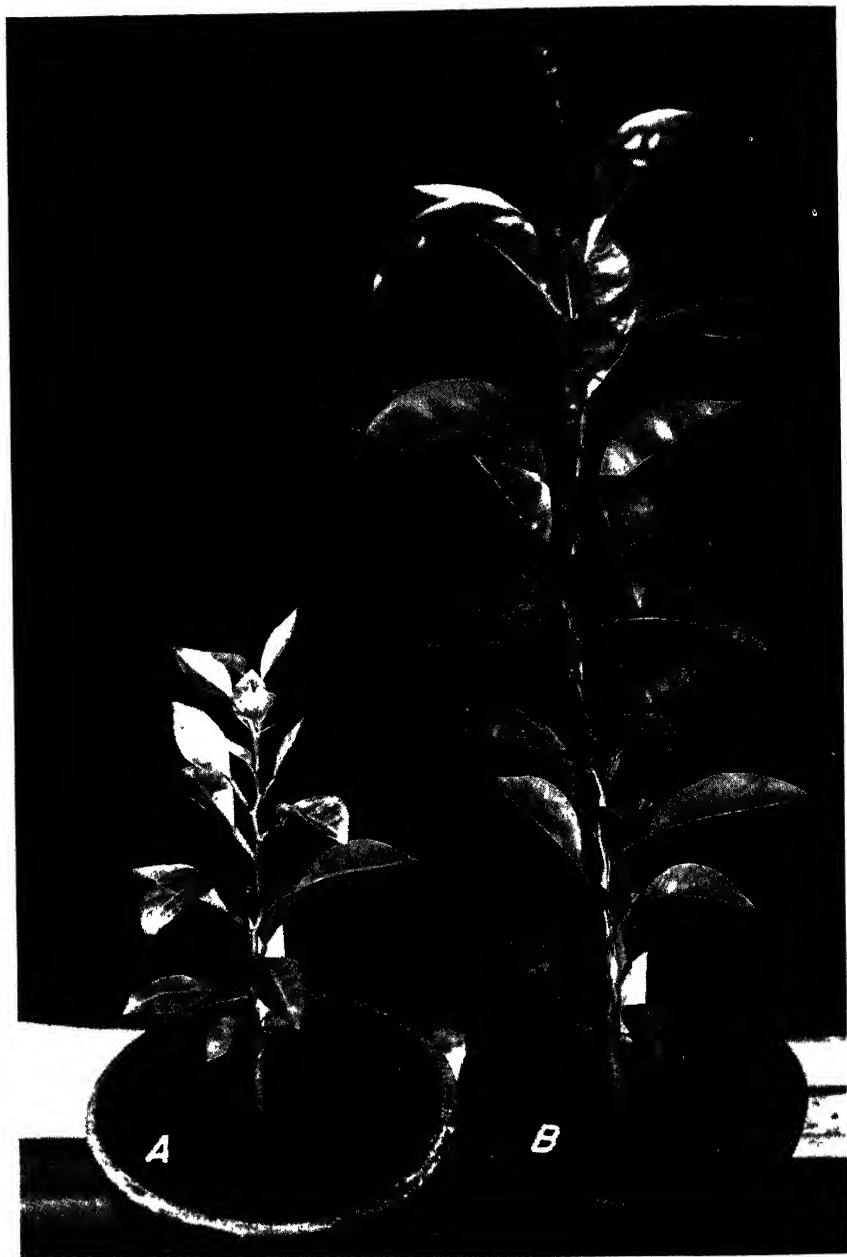
FIGURE 3.—Young sweet orange trees on sour orange rootstock photographed about 6 months after inoculation: *A*, Plant inoculated by infestation with about 200 *Aphis citricidus* from a sweet orange tree affected with tristeza; *B*, check plant infested with about the same number of aphids from healthy plants.

In two other tests different numbers of aphids collected from diseased plants were used to inoculate very small plants of the variety Barão on sour orange rootstock. Of 30 plants each infested with 1 aphid, 2 became infected; of 5 plants each infested with 5 aphids, 1 became infected; of 2 plants each infested with 25 aphids, 1 became infected; and of 4 plants each infested with many aphids, 1 became infected.

Other tests were made in which large numbers of *Aphis citricidus* were allowed to crawl from diseased orange twigs to small rapidly growing plants of various species and remain until they died. Ten to fifty plants of each of the following species were infested in this manner: *Acanthospermum hispidum* DC. (carrapicho de carneiro), *Amaranthus caudatus* L., *Brassica pe-tsai* Bailey (Chinese cabbage), *Callistephus chinensis* (L.) Nees (China-aster), *Canavalia ensiformis* (L.) DC. (feijão de porco), *Datura stramonium* (jimsonweed), *Datura* sp., *Erigeron bonariensis* L., *Euphorbia pilulifera* L., *E. prunifolia* (amendoim bravo), *Gossypium hirsutum* L. (cotton), *Lycopersicon esculentum* (tomato), *Nicotiana glauca* (tree tobacco), *N. tabacum* (tobacco), *Oxalis* sp., *Phaseolus vulgaris* L. (kidney bean, var. Scotia), *Phyllanthus corcovadensis* Muell. Arg. (quebra pedra), *Ruta* sp., *Solanum tuberosum* L. (potato), *Spinacia oleracea* L. (spinach), *Stellaria media* (L.) Cyr. (chickweed), and *Vigna sinensis* (Torner) Haask. (cowpea). No symptoms considered to be caused by the tristeza virus were discovered in any of these plants.

TESTS FOR SEED TRANSMISSION

All the seedling plants of sweet and sour oranges used in the experiments already described were grown from seeds from diseased trees or from trees that had been exposed to infection for several years. More than 1,200 plants were prepared for these experiments by grafting sweet orange seedlings on sour orange seedlings. Some of the plants were inoculated by insertion of grafts or by infestation with *Aphis citricidus* and showed the disease only after a reasonable period following inoculation. More than 425 plants, however, were held as checks or were inoculated with juice or by infestation with insects that appear not to be vectors of the causal virus. Only 1 of these plants has shown symptoms that resemble those of tristeza. Attempts to recover the tristeza virus from this plant were unsuccessful, and it was concluded that the symptoms observed were not caused by that virus.

Further tests were made with seeds collected from trees of the variety Pera on sour orange rootstock in advanced stages of disease and with seeds from trees of sour orange on their own roots which showed no symptoms of tristeza but which were surrounded by badly diseased trees of sweet orange on sour orange rootstock. Seeds of the two species were planted in flats. When the seedlings attained sufficient size, the sweet orange seedlings were grafted onto sour orange seedlings. More than 1,000 plants were prepared in this manner, placed in pots in a greenhouse, and watched for the appearance of symptoms of tristeza for 5 months. All of them remained healthy. From these results it seems probable that the tristeza virus is not transmissible through the seeds of the varieties of oranges tested.

The causal agent of the tristezalike disease of citrus in the Union of South Africa also appears not to be transmissible through the seed, since Oberholzer (*21*) found that buds from seedlings of Valencia orange and Triumph grapefruit developed normally when budded into sour orange and lemon rootstocks, whereas buds from orchard trees developed into twigs with severe symptoms of disease on these rootstocks.

VARIETAL SUSCEPTIBILITY TO TRISTEZA

The degree of susceptibility of the various species and varieties of citrus to infection with tristeza is not well known, largely because the causal virus had not been found to produce macroscopic primary symptoms on any of the plants that have been subjected to inoculation. Seedling trees of sour orange, sweet orange, rough lemon, Rangpur lime (*Citrus aurantifolia* (Christm.) Swingle, or possibly *C. reticulata* × *C. aurantifolia*), sweet lime (*C. aurantifolia*), grapefruit, "cravo" tangerine (*C. reticulata* Blanco), citron (*C. medica* L.), and trifoliate orange (*Poncirus trifoliata* (L.) Raf.) have been growing at the Estação Experimental de Citricultura de Limeira near diseased trees for more than 5 years and have shown no injury from the disease. So far as known, seedling trees of all species and varieties of citrus are immune from or highly resistant to injury, although some are known to be susceptible to infection. Thus far, damage has been observed only on trees of certain specific scion-stock combinations.

SUSCEPTIBILITY OF SCION-STOCK COMBINATIONS

The scion-stock combination injured most extensively in Argentina, Brazil, and Uruguay is sweet orange on sour orange rootstock. Other kinds of citrus on this rootstock, however, have also been reported as susceptible to injury (*2, 5, 6, 20*). In 1941 Bitancourt (*5*) listed sweet orange as susceptible to injury in Argentina and grapefruit as possibly susceptible; in 1943 he (*6*) reported sweet orange, tangerine, and grapefruit as susceptible in Brazil. Moreira (*20*), in 1942, reported the susceptibility of Pera and Bahianinha sweet orange, Marsh seedless grapefruit, Mexeriqueira tangerine, and Galacian lemon. In 1947 Bertelli (*2*) listed sweet orange, grapefruit, and mandarin as subject to injury.

During the 1946–47 season observations were made on many varieties and species of citrus growing at the Estação Experimental de Citricultura de Limeira, where a high percentage of the trees of sweet orange on sour orange rootstock were in advanced stages of disease. In these plantings most of the trees in blocks of Marsh seedless grapefruit on sour orange and on bittersweet orange (*Citrus aurantium* L.) showed symptoms characteristic of those produced by tristeza. These trees were smaller than those on sweet orange rootstock; many older leaves had dropped prematurely; in some cases weak secondary branches with small leaves had developed on some of the limbs; and the fruits were smaller and probably more numerous than on adjacent healthy-appearing trees on sweet orange rootstock. The trees had not declined in vigor or productivity so rapidly as had orange trees on sour orange rootstock. The symptoms, however,

were those that would be expected from a less severe expression of the disease. This greater resistance of grapefruit has been noted also by Bitancourt (*6*).

Orange trees of the varieties Pera and Bahianinha on grapefruit rootstock also appeared abnormal and showed many of the symptoms characteristic of tristeza. Injury was less severe than on trees of these varieties on sour orange rootstock. It has been suggested that the abnormal appearance may have resulted from a type of incompatibility between scion and stock. However, since there appears to be no evidence of a comparable incompatibility in this union in other parts of the world, it seems possible that these trees are being injured by tristeza.

In variety-test plots a short row of trees of Thornton tangelo (*Citrus paradisi* × *C. reticulata*) on sour orange rootstock had been killed by tristeza, but an adjacent row of Sampson tangelo on the same rootstock showed no evidence of injury. Differences in susceptibility of these two varieties of tangelo were reported first by Moreira (*20*) in 1942.

In experimental tests 30 or more citrus plants of each of several citrus types were prepared in 3 lots as follows: Lot 1, seedlings on their own roots; lot 2, seedlings grafted to sour orange rootstock; lot 3, seedlings grafted with sweet orange scions. Half of the plants of each lot were infested with large numbers of *Aphis citricidus* from diseased orange trees, and half were retained as checks and not infested with aphids. In these tests, which are still in progress, no evidence of disease has been observed on nongrafted trees of any species or variety, but symptoms typical of tristeza have appeared on the following scion-stock combinations: Sweet orange on Viçosa grapefruit and Doce and Melancia shaddocks (*Citrus grandis* (L.) Osbeck); and Viçosa grapefruit, Doce shaddock, Cleopatra mandarin (*C. reticulata*), Satsumelo 10–V–3, and Sunshine tangelo, all on sour orange rootstock. No evidence of disease was noted on any of the noninoculated plants of these tests.

Many scion-stock combinations appear to be free of injury from tristeza. At the Estação Experimental de Citricultura de Limeira sweet orange (vars. Pera and Bahianinha) and grapefruit (var. Marsh seedless) on sweet orange, Rangpur lime, sweet lime, "cravo" tangerine, and rough lemon have shown no injury attributed to tristeza in plantings in which most of the trees of sweet orange and grapefruit on sour orange and bittersweet orange rootstocks are now dead or severely affected with the disease. Near Limeira an 8-acre grove of trees of sour orange on sweet orange rootstock has shown no symptoms of disease, although most of the trees of the reverse combination in this area have been killed. It is known also, as reported by Bitancourt (*6*), that when trees of sweet orange on sour orange rootstock are top-worked to lemon they become resistant to injury.

In tests with small grafted plants in pots the following combinations appear to be resistant to injury: Sweet orange on Duncan grapefruit, on Cleopatra mandarin, on Satsumelo 10–V–3, and on Sunshine tangelo; and Duncan grapefruit and Melancia shaddock on sour orange. Some of these combinations, however, need further testing before

they can be definitely considered resistant. Plants of this experiment are undergoing further exposure to the disease in the field.

It seems probable that further information on varietal susceptibility may throw more light on the relation of tristeza to quick decline in California, bud-union decline in Australia, and the troubles in the Union of South Africa and Java, all of which have in common the ability to produce severe injury on trees of sweet orange on sour orange rootstock.

Marloth (*17*) found that trees of sweet orange and tangerine in Transvaal were injured when on sour orange rootstock, but that some degree of success was obtained with trees of grapefruit and tangelo on sour orange rootstock. The behavior of Sampson tangelo as a stock was found to be similar to that of sour orange. This evidence of susceptibility of Sampson tangelo as a stock, together with its resistance as a scion on sour orange in Brazil, indicates that the reaction of this variety of tangelo to the virus of tristeza may prove to be the same as that of sour orange, provided, of course, that the South African disease is caused by the same virus.

Oberholzer (*21*) stated that the commercial lemon, certain acid limes, and, to a certain degree, grapefruit react more or less in the same way as sour orange when used as rootstocks for sweet orange in the Union of South Africa.

In Java, Toxopeus (*33*) found "Japanese citron" (probably *Citrus nobilis* Lour. × *C. medica*) resistant when used as a stock for sweet orange, but when used as an interstock between a sweet orange scion and a sour orange rootstock it afforded no protection. When sweet orange, "Japanese citron," and sour orange were used as scions, interstocks, and stocks in all the possible combinations, injury occurred only in trees that had a sweet orange top and an interstock or a stock of sour orange. Terra (*32*) stated that, in Java, Cleopatra mandarin appears to make a satisfactory stock for sweet orange but that another tangerine, "Djeroek siem," was a failure as a stock for Pineapple orange. The "sour orange Peradeniya," which may or may not be a variety of sour orange, was resistant as a rootstock for sweet orange.

Trees of sweet orange on grapefruit rootstock have been reported (*30*) resistant to quick decline in California.

McAlpin, Parsai, Roberts, and Hope (*16*) listed the following combinations as susceptible to injury by bud-union decline in Australia: Sweet orange on sour orange, grapefruit, kumquat (*Fortunella* sp.), and lemon; grapefruit on sour orange and Eureka lemon; and mandarin on sour orange.

Whether the apparent differences in varietal reaction, such as the seeming differences in behavior of grapefruit as a stock for sweet orange in Brazil and in California, have any significance with respect to the causal agent in the two cases is not certain, and further observations are needed. From the limited evidence now available it may be expected that a considerable range of susceptibility to tristeza will be found among varieties of grapefruit and shaddock. This may be true also for tangerine, tangelo, and possibly other related types.

SYMPTOMLESS CARRIERS OF TRISTEZA VIRUS

Whether any recognizable effects are produced on seedling trees of any species or variety of citrus or citrus relatives by the tristeza virus

remains to be determined.   A type of vein yellowing occurring alone or accompanied by some leaf drop on isolated twigs of many trees that otherwise appear normal, however, may be caused by the tristeza virus.   The yellowing is of the type encountered on trees of sweet orange definitely affected with tristeza.   It has been found in most striking form on grapefruit in new flushes of growth.   Yellowing similar to that described on seedling trees has been observed occasionally also on trees of resistant scion-stock combinations.   It would seem possible for this type of yellowing to be caused by an accumulation of materials above a stem zone in which there has been collapse of sieve tubes in an area temporarily susceptible to injury.

It is to be expected that all types that show tristeza when grafted on sour orange or other susceptible stocks will be virus carriers as seedlings or when grafted on resistant stocks after being infected.

At present, however, there seems to be no way of determining with certainty the presence of virus in such trees other than by transmission of virus from the suspected tree to trees of scion-stock combinations known to be susceptible to injury.   In 1947 extensive attempts were made to transmit the virus from different species and varieties of citrus showing no symptoms of the disease to potted sweet orange plants on sour orange rootstock by infesting them with *Aphis citricidus*, by insertion of buds, and by cleft grafting twigs into stems of the plants to be inoculated.   The results of the first of these tests are shown in table 5.   They indicate rather definitely that the virus was present in certain citrus types in which no symptoms of the disease were recognized.   The types that showed most convincing evidence of being virus carriers are rough lemon on its own roots, "cravo" tangerine on its own roots, the Pera variety of orange on Rangpur lime, and an unidentified citrus tree on its own roots.   In this connection it is of interest that Fawcett and Wallace (*14*) have shown that the virus of quick decline is carried by trees of sweet orange that show no symptoms of the disease.

TABLE 5.—*Results of attempts to transmit tristeza virus to potted sweet orange plants on sour orange rootstock from various species and varieties of citrus exposed to infection but showing no symptoms*

Source of inoculum	Results with indicated type of inoculum					
	Aphis citricidus		Twigs		Buds	
	Plants infested	Plants infected	Plants grafted	Plants infected	Plants budded	Plants infected
	Number	*Number*	*Number*	*Number*	*Number*	*Number*
Rough lemon on own roots	0	----------	5	4	5	1
Grapefruit budded on grapefruit	0	--- -----	5	1	5	0
Eureka lemon budded on trifoliate orange	0	- -------	5	0	5	0
"Cravo" tangerine on own roots	0	----------	5	5	5	1
Pera orange budded on Rangpur lime	10	1	5	5	5	4
Sweet orange (caipira) on own roots	5	0	5	1	5	1
Citron on own roots	5	0	5	0	5	0
Rangpur lime on own roots	0	--- ------	5	0	5	0
Sour orange on own roots	10	0	10	0	10	0
Trifoliate orange on own roots	0	----------	5	0	5	0
Unidentified citrus tree on own roots	5	5	0	----------	0	----------

RELATIVE SUSCEPTIBILITY OF SWEET AND SOUR ORANGES TO INFECTION

Since trees composed of sweet orange scions on sour orange rootstock are severely injured by tristeza and are therefore of greatest economic importance in relation to the disease, it was thought worth while to attempt to determine the relative susceptibility of plants of the constituent species to infection when growing on their own roots. For these tests potted plants of each species were divided into two lots when about 10 cm. tall. In the first test one lot of each species was inoculated three times by infestation with *Aphis citricidus* from diseased orange trees at intervals of 2 weeks and the other lot was not inoculated. The aphids were allowed to feed 48 to 72 hours at each infestation and then were destroyed.

Since no reliable symptoms of tristeza have been observed on either of these species of citrus when growing on its own roots, it was necessary to graft the sweet orange onto the sour orange plants in order to determine whether infection was produced by the aphids. Ten days after the third inoculation the following graft combinations of sweet and sour oranges were made: (1) Inoculated sweet orange on noninoculated sour orange; (2) noninoculated sweet orange on inoculated sour orange; and (3) noninoculated sweet orange on noninoculated sour orange.

In the second test small plants of sweet and sour oranges about 10 cm. tall were inoculated by infestation with *Aphis citricidus*. Two days after inoculation the aphids were killed and the three graft combinations listed for the first test, as well as inoculated sweet orange on inoculated sour orange, were made.

The results of these tests (table 6) show that small sour orange plants are susceptible to infection and that when used as stocks they transmitted the infection to noninoculated sweet orange scions. The results indicate strongly also that the sour orange plants were less susceptible to infection than the sweet orange plants. Since as a rule plants increase in resistance to virus diseases as they become larger, it seems probable that larger plants of sour orange may prove to be still more resistant.

TABLE 6.—*Results of grafting inoculated and noninoculated small seedling sweet orange plants on inoculated and noninoculated plants of sour orange*

[Inoculation by infestation with *Aphis citricidus*]

Test No.	Graft combination used	Plants tested	Plants showing disease
		Number	*Number*
1	Inoculated sweet orange on noninoculated sour orange	38	22
	Noninoculated sweet orange on inoculated sour orange	36	9
	Noninoculated sweet orange on noninoculated sour orange	20	0
2	Inoculated sweet orange on noninoculated sour orange	14	5
	Noninoculated sweet orange on inoculated sour orange	15	1
	Noninoculated sweet orange on noninoculated sour orange	18	0
	Inoculated sweet orange on inoculated sour orange	8	5

EVALUATION OF CONTROL MEASURES USED IN BRAZIL

Control measures for tristeza in Brazil have been of two kinds: (1) Those designed to save trees of susceptible scion-stock combinations from destruction and (2) those designed to avoid future losses in new plantings.

The measures that have been used to save trees of susceptible scion-stock combinations already planted consist of (1) inarching with resistant stocks; (2) mounding the soil to a level higher than the graft union to stimulate the production of roots from the scion; (3) top working with lemon; and (4) encouraging growth of sour orange shoots from the trunk or root system of the stock with the hope that these shoots will supply the carbohydrates necessary to replace those that are normally supplied by the sweet orange top.

The success of the method of control involving inarching depends largely on the size and kind of tree involved, on the time of infection of the treated tree in relation to time of inarching, and to some extent on environmental factors. In general, large trees, especially if inarched after they become diseased or shortly before, have not given encouraging results. However, after tristeza appeared in the grove at Fazenda São Sebastião, Caratuatatuba, 20,000 trees of Marsh seedless grapefruit budded on sour orange stock and varying in age from 6 to 12 years were inarched with resistant rootstocks with a high degree of success. Over 90 percent of the treated trees recovered or were maintained in a satisfactory condition. This grove is located near the coast, where high temperatures and humidity favor almost continuous growth.

In an orange grove belonging to the same firm, but located on the plateau, where the climate is drier and the temperatures lower, 27,000 trees of the variety Bahianinha and 5,000 trees of the variety Hamlin on sour orange rootstock, ranging in age from 6 to 12 years, were inarched with resistant stocks. Although about 70 percent of the young trees used in inarching became established, the inarched trees have not regained their former vigor and productivity.

In other orange groves in which inarching has been practiced some trees appear to have been benefited, but in areas where the disease is prevalent few inarched trees that have been maintained in a highly productive condition have been observed. It seems probable, however, that trees inarched well in advance of infection so that they would be largely on resistant roots before infection occurred could be saved.

With small trees there is evidence that control by inarching is more successful, and it is probable that most young groves could be saved from destruction by inarching with resistant varieties if inarching were started before invasion or in the early stages of invasion of the groves by the disease. In practice, the method has not been used extensively, probably largely because of the cost.

From the observations thus far trees of grapefruit appear to have given better results from the inarching method of control than have those of sweet orange. This may be due, in part at least, to a slower rate of decline of trees of grapefruit after infection, thus affording more

favorable conditions for the young trees used for inarching to become established.

The type of citrus used as rootstocks in inarching appears to be important in the success of the method. Observations made by A. J. Rodriegues [6] on three types of rootstocks indicated that Rangpur lime and sweet lime gave somewhat better results than sweet orange (caipira) in groves in which tristeza was present.

Mounding of the soil around the trunks of diseased or healthy trees of susceptible combinations to stimulate the growth of roots from the scion has not been tried extensively in Brazil, mainly because as a rule the trees are budded so high that large mounds are required to cover the base of the scion. Also some difficulty has been experienced in obtaining root development from the scion. The method may prove more effective where the trees are budded lower and where root rot is not a serious problem.

Top working susceptible orange trees with lemon has been very effective in controlling tristeza where the trees were not too large or too badly damaged when the top working was done. The practicability of this method of control depends, of course, largely on whether there is a market for the resulting lemon crop. Partly because of market limitations and demands this method of control has been used only to a limited extent.

In the Fazenda São Carlos near Palmeiras encouraging the growth of shoots from the trunk and root system of sour orange is being tested commercially on a large scale in a grove of 30,000 orange trees in which tristeza is just becoming established. Whether these shoots will supply the deficit in carbohydrates in the stock caused by the disease and thus prolong the productive life of the tree remains to be determined. Some trees have been observed with sour orange sprouts 3 to 5 years old growing from the trunk and from the root system, but the sweet orange tops in all cases have continued to decline.

Since a very high percentage of the orange trees of Brazil of scion-stock combinations susceptible to injury by tristeza have been destroyed or rendered of little value, the problem of control of the disease there is largely one of replanting with resistant trees and of choosing the most suitable resistant citrus types to replace sour orange as a rootstock. Brieger and Moreira (*8*) have presented extensive data on the performance, over a period of years, of 12 citrus types as rootstocks for the Marsh seedless variety of grapefruit and for the Pera and Bahianinha varieties of sweet orange. The types that have given best results in these tests are sweet orange (caipira and laranja lima), rough lemon, Rangpur lime, and sweet lime. These are the types now being used most extensively in Brazil as rootstocks for the commercial varieties of sweet orange.

It seems evident that it will be necessary to discard sour orange as a rootstock for varieties of sweet orange and perhaps for grapefruit and some varieties of tangerine and related types in all parts of the world where tristeza occurs unless types of sour orange more tolerant of the disease are obtained. Because of danger of spread of the disease to districts now free of infection, it may be advisable also to begin to

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[6] Unpublished information communicated to A. S. Costa.

limit the use of sour orange as a rootstock in many districts where the disease has not been introduced.

Chiefly because of its resistance to certain types of phytophthora rots the loss of the sour orange will be felt keenly, especially in those areas that do not have open, well-drained soils. The spread of tristeza has emphasized the need for greater resistance to root rot and crown rot organisms in rootstocks that are resistant to tristeza. A very valuable contribution to the search for such resistance has been made already by Rossetti (*26*), who presented evidence that there is a considerable range of resistance to species of *Phytophthora* in seedlings of several varieties of sweet orange. Her work encourages the hope that in time rootstocks of sweet orange, and perhaps of other types, that have a higher degree of resistance to root rot organisms may be found. The investigations stimulated by the ravages of tristeza may be expected to add materially to the knowledge of the value of many types of citrus for use as rootstocks.

DISCUSSION

Tristeza of citrus is characterized largely by failure to produce primary symptoms on the foliage of any known host plant and by the production of severe effects only on trees of certain scion-stock combinations, the best known of which is sweet orange on sour orange rootstock. Neither of the component species of the latter combination is known to be susceptible to injury when on its own roots, and no injury is evident on trees composed of sour orange tops on sweet orange roots. Scion-stock relations in some respects similar to this have been reported by Rawlins and Parker (*24*) for buckskin disease of cherry, by Raleigh (*23*) for potato latent mosaic, and by Gardner, Marth, and Magness (*15*) for a disease of apple which follows the grafting of certain apple scions on Spy 227 rootstock.

Those symptoms so far described for tristeza are secondary and are of a type produced readily by a partial or complete girdle. Evidence indicates, in fact, that a form of girdling is responsible for the known symptoms of the disease. Schneider, Bitancourt, and Rossetti (*29*) have shown that in trees affected by tristeza sieve tubes and companion cells below the union of scion and stock become necrotic. Injury of this type would be expected to lead to an excessive accumulation of metabolites in the top of the tree and to a food deficiency in the root system. Thus, it might be expected that the first symptoms of the disease would be associated with the accumulation of excessive amounts of carbohydrates and other products in the tops of the trees and that the later symptoms might be influenced to a greater degree by injury to the root system resulting from deficiency of food materials.

The sequence of symptoms produced in young trees inoculated with tristeza virus in the field followed this expected pattern. Leaves of infected trees 4 to 6 months after inoculation took on a dull appearance, often with a tendency toward vein yellowing. This first noticeable injury did not appear to be due to root deterioration, since no evidence of starch depletion or root decay was detected in tests made shortly after symptoms began to appear. Symptoms somewhat similar, but characterized by more vein yellowing, were produced in about the

same length of time on check trees on limbs around which a wire had been wrapped.

Also similar secondary symptoms of vein yellowing and chlorosis were observed on potted orange plants heavily infested with cottony-cushion scale (*Icerya purchasi* Mask.). This yellowing is thought to have been associated with injury to the phloem by insect-produced toxins.

The peculiar scion-stock relation in the production of symptoms of tristeza is of special interest. It seems probable that for the production of injury in the species and varieties of citrus so far observed (1) the scion must be of a species or variety which produces relatively large quantities of virus but which is tolerant of the virus and of products resulting from its presence and (2) the rootstock or the interstock must be of a species or variety which is capable of producing relatively low concentrations of the virus and which is highly sensitive to the virus or to products resulting from its presence.

Plants of a variety or species capable of producing high concentrations of the virus or of virus toxin and having phloem sensitive to injury by it would be expected to show symptoms on their own roots and would be valuable as test plants for use in the study of the disease. As yet no plant of this type has been discovered.

Information available thus far seems to indicate that the sweet orange is relatively susceptible to infection and that it may be capable of supporting relatively high concentrations of virus, but that the sour orange is less susceptible to infection and may have lower concentrations of virus. It seems probable, therefore, that infection of a tree composed of a sweet orange scion and a sour orange rootstock would be followed by multiplication of the virus in the sweet orange tissue and by the production of relatively high concentrations of virus in the top. Since it has been demonstrated that viruses that enter the phloem move rapidly in the direction of translocation of elaborated food products, it seems probable that the toxic principle, whether it be a virus or a toxin resulting from the presence of the virus, would move rapidly and in considerable quantity from the scion into the stock and that the concentration of injurious material in the sour orange, particularly immediately below the union between scion and stock, would be much higher than could be reached as a result of virus increase in a sour orange tree.

It is also conceivable that virus multiplication in a sour orange root-stock might be increased greatly by a sweet orange top if the assumption is made that virus increase takes place at the expense of materials carried in the phloem and that materials are carried in the phloem as a result of mass movement of the liquid phloem content. In this case it would be expected that virus precursors built up in the sweet orange would move from the sweet orange to the sour orange and that the phloem content of the sour orange stock immediately below the union with the scion would be more characteristic of the phloem content of sweet orange than of sour orange. Thus by virtue of these materials increase might occur, and the virus or virus toxin might reach concentrations sufficiently high to cause collapse of sieve tubes.

There is evidence in the case of at least one virus to support the idea that materials can move from a susceptible virus-infected·scion and

produce symptoms of disease on a resistant stock. When a scion of Turkish tobacco infected with a virus which is believed to be a mutant of the sugar-beet curly top virus and which produces marked yellowing of veins of leaves of Turkish tobacco is grafted onto a plant of *Nicotiana glauca*, the first leaves of the new shoots from the stock have yellow veins. This condition persists as long as the shoots from the stock are in a position to receive food material from the scion, but when they grow and presumably become independent of the scion for their food supply they no longer produce leaves with yellow veins. For all practical purposes *N. glauca* is immune from this virus, for when the scion is removed the stock no longer produces leaves with yellow veins and soon the virus is lost.

On the basis of these concepts theories may be advanced to explain certain anomalies such as injury in trees having an intergraft of sour orange between a sweet orange top and a resistant root and the resistance of trees with an intergraft of sweet orange between a lemon top and a sour orange rootstock. In the first instance the virus would move from a susceptible sweet orange, possibly highly charged with virus, into sour orange tissue and collapse of sieve tubes would be expected in the sour orange regardless of the type of tissue below the sour orange interstock. In the second instance, although movement into the sour orange stock would be from sweet orange, the materials moving from the sweet orange into the sour orange probably would be largely those manufactured by lemon. Since it seems probable that lemon is a relatively unfavorable medium for virus increase, only low concentrations of toxic material at most would move from the lemon through the sweet orange interstock into the sour orange stock. These concentrations might well be too low to cause serious injury to the phloem.

The reason for the failure of tristeza virus to produce appreciable injury on any species or variety of citrus growing on its own roots is of considerable interest from both a scientific and a practical viewpoint. The most obvious explanation of this curious fact would appear to be that no species or variety known at present is able to produce sufficient concentrations of virus or of virus toxin within its own tissues to cause appreciable injury to its own phloem. On the basis of this theory it seems probable that there may eventually be found citrus species and varieties on their own roots that show five more or less well defined relations to the virus, only three of which have as yet been encountered with any degree of certainty. These relations may be indicated as follows:

(1) The plant may permit little or no virus increase but possess phloem tissue very susceptible to injury by the virus or toxin. Sour orange, Sampson tangelo, and some true lemons show evidence of such a relation.

(2) The plant may permit production of moderate concentrations of virus or toxin and possess phloem tissue only moderately susceptible to injury. Some varieties of grapefruit and shaddock may have this relation.

(3) The plant may permit the production of high concentrations of virus and possess phloem tissue highly resistant to injury. Sweet

orange, and perhaps Rangpur lime, rough lemon, and some varieties of tangelo and mandarin, may have this relation.

(4) The plant may permit the production of little or no virus and possess phloem tissue that is tolerant of high concentrations of virus or toxin resulting from the presence of virus. Varieties showing this relation are not known, but possibly some varieties of lemons or other resistant types have such a relation. A variety of this kind would be expected to be resistant both as a stock and as a scion for other citrus varieties.

(5) The plant may permit the production of relatively high concentrations of virus and possess phloem very sensitive to injury. Such a plant, of course, should show symptoms as a seedling and would be of value in experimental work.

On the basis of these concepts it would be expected that any given scion-stock combination will prove to be resistant to injury by tristeza if the stock is of a variety that on its own roots is capable of producing a virus concentration as high as that normally produced by the scion variety on its own roots or higher. Also, it may be expected that any given scion-stock combination will prove to be susceptible if the stock is of a variety that on its own roots produces a lower concentration of virus or toxin than that produced by the scion variety unless the stock variety falls in class 4. With many varietal combinations it is possible that the degree of injury to the tree may be to some degree proportional to the difference in these respective concentrations in stock and scion varieties.

These postulated relations provide a basis for a logical explanation of the seemingly contradictory observations that grapefruit is susceptible to injury as a scion when it is on a sour orange rootstock and apparently susceptible to injury also as a stock when the scion variety is sweet orange, whereas it is less susceptible to injury when both stock and scion are grapefruit. Grapefruit trees on sour orange stocks are injured less than sweet orange trees on sour orange stocks, indicating that grapefruit produces a smaller amount of toxic materials than sweet orange. Also trees of sweet orange on grapefruit stocks are injured less than trees of sweet orange on sour orange stocks, indicating that grapefruit is less susceptible than sour orange as a stock. Thus a lower concentration of virus or toxin in grapefruit as a scion together with greater resistance of grapefruit as a stock may result in resistance to injury in a tree composed of a grapefruit top on a grapefruit root, even though grapefruit may be susceptible to injury both as a stock and as a scion in certain other scion-stock combinations.

At the Instituto Agronômico at Campinas, Brazil, the concepts listed have been the basis for planning some of the tests having for their objective the determination of relative tristeza-resistance values of citrus species and varieties both as scions and as stocks. In addition to the direct testing of numerous types of citrus as rootstocks for sweet orange, grapefruit, and other types, an indirect test in which numerous citrus types are grafted onto sour orange is being made. It is expected that, in general, the degree of injury shown by these types on sour orange will be a relative measure of the concentration of virus or virus toxin that the scion variety is able to tolerate without injury. Scions that show severe injury on sour orange rootstock may prove to

be resistant as rootstocks for varieties that show less injury as scions on sour orange rootstock. Thus it may be that varieties capable of producing high concentrations of virus will prove to be among the best rootstocks from the standpoint of resistance to injury by tristeza.

The relation of tristeza in Brazil to similar disorders in other places, notably the Union of South Africa, Argentina, Uruguay, Java, Australia, and southern California, has been the subject of considerable speculation. However, in some cases more evidence on which to base definite conclusions is needed. Tristeza in Brazil appears to be identical with podredumbre de las raicillas in other South American countries. There are marked similarities also between tristeza and the disorders which occur on sweet orange varieties on sour orange rootstock in the Union of South Africa and Java. Olmo and Moreira (*22*) emphasized the similarities between tristeza in South America and quick decline in California. Schneider (*28*) pointed out that almost identical types of phloem necrosis are found in trees infected with tristeza and with quick decline; and Meneghini (*19*) emphasized similarities in starch depletion in the sour orange rootstock.

Despite these striking similarities certain variations in the effects produced in different parts of the world suggest that the diseases are not identical in all instances. Some of the observed differences, such as the reported resistance of trees of sweet orange on grapefruit rootstock to quick decline and the failure of such trees to develop normally in certain districts in which tristeza is present, may be due to varietal or other differences. Variation in certain symptoms, for example, may be related to soil and climatic conditions. It is not clear, however, that all of the observed variations are the result of varietal and environmental influences.

Toxopeus (*33*) reported that in Java buds from sweet orange died in 8 to 12 months after being placed in sour orange stocks, and Oberholzer (*21*) stated that in the Union of South Africa young trees formed by budding Valencia orange into susceptible rootstocks began to exhibit extensive root decay, chlorosis, and general decline at a very early age, usually 1 to 3 months after budding. Such rapid decline or killing has not been noted in Brazil after the budding of either diseased or healthy sweet orange buds into sour orange rootstock. In experimental tests buds from sweet orange trees affected with tristeza budded into healthy sour orange rootstocks developed more or less normally at first and after 12 months they showed a reasonably high degree of vigor. In nursery trees inoculated with the tristeza virus in Brazil by insertion of buds from diseased trees, the incubation period of the disease, as measured by leaf discoloration, was as short as 4 months, in comparison with about 15 months observed by Fawcett and Wallace (*13*) for quick decline in California. Also in trees affected with tristeza (*2, 7, 25*) and with quick decline (*11*) there appear to be differences in the amount of starch found in the bark of sweet and sour oranges in the vicinity of the graft union and in that found in the roots of the sour orange rootstock in certain stages of development of the disease.

If these differences prove not to be due to varietal and environmental influences, they indicate that tristeza, as it is known in Brazil, differs in some respects from similar disorders in Java, California, the

Union of South Africa, and perhaps Australia. If these disorders are not caused by the same virus, then it must be assumed on the basis of information now available that they are caused either by closely related viruses or by virus strains that vary in virulence and possibly in other characteristics. The latter alternative seems the more tenable at present.

If tristeza originated in the Union of South Africa as has been suggested, all the strains of the causal virus would be expected to occur there provided no mutation has occurred in areas more recently invaded. If virus was carried from southern Africa to other parts of the world, as seems likely, it would be expected that not all of the strains would necessarily be introduced into any one place at a given time. The characteristics of the disease produced in the different countries would be expected to vary, therefore, according to the strain or strains of the virus introduced.

If strains of a single virus or even related viruses cause the various disorders and if these strains or related viruses differ in their ability to attack different species and varieties of plants, as is known to be the case with certain other virus diseases, it seems very important to obtain as soon as possible information on the reaction of a wide range of varieties and species of citrus in all parts of the world where the disorders occur. This is true especially of varieties of grapefruit, mandarin, lemon, tangelo, tangerine, and any other types that may be considered for use as rootstocks to replace sour orange.

SUMMARY

Tristeza of citrus, first observed in Argentina in 1930 or 1931 and in Brazil about 1937, now occurs in most of the commercial citrus districts of Argentina, Brazil, and Uruguay. The same or similar disorders, all characterized by ability to cause serious damage to varieties of sweet orange on sour orange rootstock, have been reported from the Union of South Africa, Java, southern California, and Australia.

In experimental work at Campinas, Brazil, transmission of tristeza was not obtained by means of juice from diseased sweet orange plants. Of 91 sweet orange plants inoculated by establishing on them dodder (*Cuscuta* spp.) that had grown on diseased plants, 1 plant inoculated by means of *C. subinclusa* became infected and 1 inoculated by means of *Cuscuta* sp. showed symptoms resembling tristeza, indicating that the disease may be transmissible to a low percentage of plants by this means.

The disease was transmitted to 10 varieties of 'sweet orange on sour orange rootstock growing under field conditions by the insertion of buds from diseased trees and to sweet orange plants on sour orange rootstock in the greenhouse by the insertion of buds and twigs from diseased trees.

The causal virus was transmitted to small plants under greenhouse conditions by infesting them with the oriental citrus aphid (*Aphis citricidus*) from diseased plants, confirming the transmission results of Meneghini with this insect. A low percentage of plants infested with single aphids became infected, but high percentages of infection were obtained only with large numbers of insects. In limited tests a period of starvation prior to feeding on diseased plants appeared not

to increase the ability of the insects to transmit the virus. Transmission was obtained with aphids only after they had fed 60 minutes or longer on diseased plants.

· No transmission was obtained by means of any of 10 other species of insects, including 5 of aphids, 4 of leafhoppers, and 1 of white fly, after they had fed on diseased sweet orange trees.

A number of species and varieties of annual plants inoculated with juice from infected sweet orange trees by establishment of dodder from such trees and by infestation with *Aphis citricidus* developed no symptoms that indicated transmission of the virus.

In tests involving more than 1,400 plants the disease was not transmitted through the seeds of sweet or sour orange.

No symptoms known to be caused by tristeza have been observed on any variety of citrus or citrus relative on its own roots, although it is thought possible that certain types of vein yellowing observed occasionally on isolated twigs of trees of grapefruit and many other types of citrus may be caused by the disease. The scion-stock combinations that have been observed to be injured are the following: Sweet orange, Marsh seedless grapefruit, Mexeriqueira tangerine, mandarin, Thornton tangelo, and Galacian lemon on sour orange rootstock, and probably sweet orange on grapefruit rootstock. Results from inoculation of small plants by means of *Aphis citricidus* have indicated that the following scion-stock combinations may be susceptible to injury under greenhouse conditions: Sweet orange on Viçosa grapefruit and Doce and Melancia shaddocks; and Viçosa grapefruit, Doce shaddock, Cleopatra mandarin, Satsumelo 10–V–3, and Sunshine tangelo on sour orange rootstock.

In tests in which small seedlings were inoculated by means of *Aphis citricidus*, sweet orange seedlings appeared to be more susceptible to infection than sour orange seedlings.

The virus was transmitted to small sweet orange plants on sour orange rootstock from trees of the following scion-stock combinations that showed no evidence of disease: Pera variety of sweet orange on Rangpur lime, rough lemon on its own roots, "cravo" tangerine on its own roots, and an unidentified citrus tree on its own roots.

Methods of control that have been used in citrus plantings in Brazil are (1) inarching with resistant stocks, (2) mounding of the soil around the base of the trees to induce the production of a root system from the scion variety, (3) top working with lemon, and (4) encouraging the growth of sour orange shoots from the stock with the hope that they will supply to the root system the necessary food materials to prolong the productive life of the trees. For various reasons none of these has proved satisfactory for general use. Since a high percentage of the bearing trees of Brazil were on sour orange rootstock and most of these have been killed or rendered valueless, control measures in the future will consist chiefly in making new plantings with trees of scion-stock combinations that are resistant to injury. Thus far the most satisfactory rootstocks have proved to be sweet orange, rough lemon, Rangpur lime, and sweet lime.

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INHERITANCE OF A GENE FOR NEAR-WILT RESISTANCE IN THE GARDEN PEA[1]

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INTRODUCTION

Fusarium wilt of garden pea (*Pisum sativum* L.) was first recorded by Jones and Linford (*2*)[3] in 1925. In a later paper Linford (*3*) described the disease and the causal organism as *Fusarium orthoceras* (App. and Wr.) var. *pisi* Linford. He pointed out that some varieties are very resistant to the disease. Wade (*10*) studied the inheritance of the resistance to wilt and concluded that it was controlled by a single dominant gene. Walker (*11*) found that resistance to the disease was present in many varieties, absent in others, and variable in different seed lots of a few varieties. He pointed out that development of resistant varieties would involve only· pure-line selection in some varieties while others would require selection from the progeny of hybrids with a resistant parent. Work along both these lines by breeders in the following years provided a range of wilt-resistant varieties for canning, many of which were adopted widely by the trade.

When wilt-resistant varieties came into general use, a closely similar disease was discovered (*5, 13*) which had been masked apparently by the more rapidly developing wilt disease. Snyder and Walker (*7*) described the new disease in detail in 1935 and called it near-wilt because of the similarity of its symptoms to those of wilt. They designated the causal organism as *Fusarium oxysporum* Schl. f. 8 Snyder. The wilt and near-wilt organisms were designated later by Snyder and Hansen (*6*) as *Fusarium oxysporum* f. *pisi* (Linford) race 1 Snyder and Hansen and *F. Oxysporum* f. *pisi* (Snyder) race 2 Snyder and Hansen, respectively.

Most varieties resistant to wilt were found to be susceptible to near-wilt (*8*). In a few, e. g., Rogers K and Horal, a moderate degree of resistance was exhibited but was not readily fixed by inbreeding. It appeared to be polygenic in inheritance and markedly influenced by

[3] Numbers in parentheses refer to Literature Cited, p. 249.

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environment. One line of breeding material was discovered which appeared to have practically complete field resistance to near-wilt. A selection of this line was increased and eventually introduced as a near-wilt resistant variety under the name Delwiche Commando in 1945 (*1*). In order to study critically the nature of inheritance of this newly discovered type of resistance to near-wilt and to transfer it to other varieties, crosses were begun in 1942. This paper is an account of the inheritance of resistance to near-wilt, a preliminary report of which has already been made (*12*).

METHODS AND MATERIALS

Field tests were made on soil naturally infested with the near-wilt organism, except in 1947. From 1938 to 1942, inclusive, a near-wilt plot near DeForest, Wis., was used. From 1943 to 1946, inclusive, trials were conducted near Waupun, Wis. This plot was in a field mapped for severity of infestation in 1941 when most plants in a commercial planting became severely diseased. In 1943, plantings were made on May 13 in rows 4 feet apart at about 8 seeds per foot of row. In each row, 6-foot strips of Wisconsin Perfection, a variety resistant to wilt but susceptible to near-wilt, were alternated with 18-foot strips devoted to other varieties, strains, or hybrids. Ordinary woven-wire fence was erected along the row to support the plants. Cultural practices commonly used to produce a canning crop were employed. At appropriate times, usually between the early-pod and canning stages, the plants were classified as resistant or susceptible to near-wilt. When conditions were favorable for disease development, as was the case in 1943, no trouble was encountered in making determinations.

Under conditions favorable to disease development susceptible plants, with the exception of a few scattered escapes, yellowed, wilted, and died rapidly. In the field, the xylem was generally brick red in color by the time the plants wilted. Under less favorable conditions the cause of the death of a plant was often questionable, and under some conditions susceptible plants showed no signs of disease at any time.

Plantings in 1944 were made on May 15 and 16 in the same manner as in 1943. No fence was used. Check plantings were spaced as in 1943 except where the hybrid did not involve Wisconsin Perfection; then the susceptible parent was alternated with the latter variety. Dates of planting and the methods of culture in subsequent years were similar to those of 1944.

In the greenhouse, 12 seeds were planted in each of 8 rows in sterilized quartz sand contained in metal pans 3 x 12 x 18 inches in dimension. The plants were watered as required with a balanced nutrient solution. The pans were placed in Wisconsin soil-temperature-control tanks held at $21° \pm 2° C$. The air temperature was more variable than the sand temperature, but it averaged about 22° C. At 7 to 10 days after seed was sown the plants were removed and the taproots were clipped to a uniform length of 1 inch while the whole root system was immersed in a heavy suspension of spores and mycelial fragments of the near-wilt fungus. The plants were replaced in the

pans after inoculation. Inoculum was grown on a modification of Czapek's solution [4] in 6-liter flasks placed on a mechanical shaker.

Disease ratings were made approximately 3 weeks after inoculation. The segregating lines were compared with resistant Delwiche Commando and susceptible Wisconsin Perfection in each pan.

EXPERIMENTAL RESULTS

RESISTANT PARENT

In the course of screening several hundred breeding progenies for resistance to near-wilt it was noted that one line (738–49) remained practically free from near-wilt on the DeForest plot, while such wilt-resistant varieties as Wisconsin Perfection succumbed completely. When the former line was increased it maintained the same high degree of resistance and it was eventually released as Delwiche Commando. Line 738–49 was derived from a cross between Pride, a near-wilt susceptible variety, and a breeding line, 732–66–1. The latter was an F_3, progeny from a cross between two lines of Admiral. A mass increase of 732–66–1, tested in 1938, yielded 75 percent resistant and 25 percent susceptible plants. Of seven single plants selected from the last-mentioned resistant group tested on near-wilt soil, the progeny of one contained 100 percent susceptible plants, and those of five segregated at ratios closely approximating 3 resistant to 1 susceptible. Three single-plant selections from the last-mentioned segregating lines segregated in the same ratio in 1940. It was apparent, therefore, that the near-wilt resistance in 738–49 had been derived from the 732–66–1 parent. Furthermore, there was a strong indication that the resistant character in this line was controlled by a single dominant gene.

FIELD TRIALS WITH HYBRID PROGENIES

In order to transfer this type of resistance to other canning types and to determine its mode of inheritance, 738–49 was crossed with numerous other varieties. The initial study of inheritance reported herein was concerned primarily with crosses between 738–49 and four wilt-resistant, near-wilt susceptible varieties, Wisconsin Perfection, Perfected Wales, Merit, and Penin. The last two varieties had been derived from a cross between Horal and Prizewinner. They each contained a moderate amount of resistance to near-wilt characteristic of that which, as already mentioned, was noted earlier in Horal. This latter type of resistance was not linked with the type of resistance in 738–49, but, as will be noted later, its expression in hybrid material sometimes obscured that of the resistant character from 738–49.

In the fall of 1942, five sister lines of 738–49 designated as 738–49/I, II, III, IV, and V were crossed with Wisconsin Perfection, Perfected Wales, and Merit and 738–49/I was crossed with Penin. The F_1 generation of these crosses was grown in the greenhouse in the spring of 1943. From the resulting seed, F_2 progenies were grown on the Waupun plot. The near-wilt disease appeared during the first week in July. On July 16, all the plants in Wisconsin Perfection controls were wilted or dead except for a few in one corner of the plot. Most.

[4] KNO_3, 3.0 gm.; KH_2PO_4, 1.0 gm.; $MgSO_4$. $7H_2O$, 0.5 gm.; KCl, 0.5 gm.; $FeSO_4$, trace; dextrose, 30 gm.; water, 1,000 ml.

of the escapes had definite symptoms of near-wilt by July 20 although many of them were not dead. Progeny tests with seed from the occasional healthy survivors yielded plants 100 percent of which became diseased when tested in 1944, showing that the healthy plants in the controls were escapes. Four counts of diseased plants were made, the wilted ones being discarded at each reading. The results are presented in table 1.

TABLE 1.—*Occurrence of near-wilt in F_2 progenies from crosses between resistant and susceptible lines*

| Cross No. | Parents | Plants wilted on date indicated | | | | Total plants wilted | Total plants healthy | Chi-square for goodness of-fit to 3:1 ratio |
		July 9	July 12	July 16	July 20			
		Number	*Number*	*Number*	*Number*	*Number*	*Number*	
C 125	Perfected Wales × 738–49/I	5	5	36	29	75	197	0.83
C 129	Perfected Wales × 738–49/II	2	4	7	0	13	47	.20
C 132	Perfected Wales × 738–49/III	7	1	10	0	18	33	2.36
C 135	Perfected Wales × 738–49/IV	3	2	11	0	16	30	1.86
C 138	Perfected Wales × 738–49/V	1	0	18	2	21	58	.04
C 126	Merit × 738–49/I	6	0	5	10	21	94	2.44
C 130	Merit × 738–49/II	4	1	9	0	14	25	1.92
C 133	Merit × 738–49/III	12	0	4	3	19	28	5.17*
C 136	Merit × 738–49/IV	0	0	8	1	9	28	(¹)
C 139	Merit × 738–49/V	0	0	3	2	5	27	1.04
C 127	Penin×738–49/I	11	1	69	3	84	150	14.25**
C 128	Wisconsin Perfection × 738–49/I	23	7	49	5	84	234	.27
C 131	Wisconsin Perfection × 738–49/II	1	2	10	0	13	45	.09
C 134	Wisconsin Perfection × 738–49/III	7	0	8	0	15	43	(¹)
C 137	Wisconsin Perfection × 738–49/IV	10	4	7	0	21	41	2.15
C 140	Wisconsin Perfection × 738–49/V	15	4	4	0	23	59	.26
	738–49/I	0	0	4	1	5	² 500	
	738–49/II	0	0	16	0	16	² 500	
	738–49/III	0	0	7	1	8	² 500	

*Significant, 5-percent level; **significant, 1-percent level.
¹ Value for Chi-square less than 0.01.
² Approximate number.

Three sister lines of 738–49, one of which was later released as Delwiche Commando, were grown along with the F_2 progenies. The 738–49 lines were highly resistant to the disease except for 1 to 3 percent of the plants which wilted and died with symptoms that resembled but were not entirely typical of those of near-wilt. One of of the 738–49 lines is shown in figure 1, adjacent to the susceptible Wisconsin Perfection control. Fourteen of the 16 F_2 progenies showed a good fit to the ratio of 3 healthy plants to 1 diseased. When the crosses were made, the seeds from all pods that set in a given cross were bulked. An occasional pod may have resulted from self-pollination of the susceptible parent. This may account for the poor fit in C127, which was due to an excess over the expected number of susceptible plants.

Selections were made from the surviving F_2 plants in 1943. Some of these were planted in the greenhouse during the following winter. The resulting F_4 generations and the remnants of F_3 progenies were planted in the disease plot near Waupun in 1944. Near-wilt did not appear in the plot until the second week of July. Disease notes were taken on July 17, 20, and 24. The reactions of the F_3 families in 1944
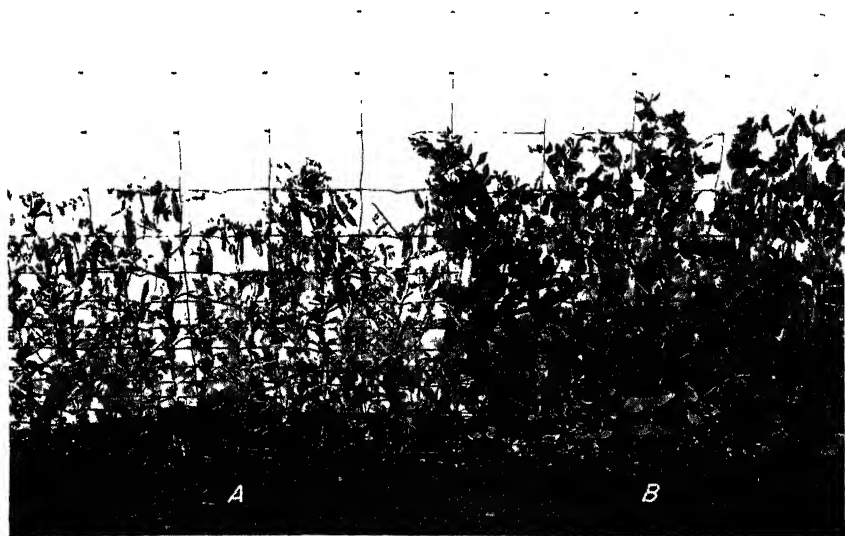
FIGURE 1.—Peas grown on soil naturally infested with *Fusarium oxysporum* f. *pisi* race 2 near Waupun, Wis., in 1943: *A*, Wisconsin Perfection, susceptible; *B*, 738–49/I, resistant, a sister line of which was increased and released as a new near-wilt resistant variety, Delwiche Commando.

are summarized in table 2. Since the susceptible plants were discarded in the F_2, segregation would be expected to be in a ratio of 1 resistant progeny to 2 segregating. According to the Chi-square test, 15 of the 16 crosses showed a good fit to the 1:2 ratio. There were a few progenies that were completely susceptible, indicating that an occasional susceptible plant escaped in 1943, as has already been pointed out with respect to the susceptible controls. There was a total of 10 such progenies, 3 in one cross, 2 in 3 other crosses, and 1 in a sixth cross. In each of these Merit or Penin was the susceptible parent.

The reaction of F_4 families from heterozygous F_2 plants in the various crosses may be seen in table 3. Excluding cross C127, 13 of the 15 crosses showed a good fit for a 1:2:1 ratio, and 53 of the 59 groups of families from individual F_2 plants were a good fit to a 1:2:1 ratio. In the C127 cross which had Penin as the susceptible parent there was a large number of very late-maturing segregates. Since near-wilt symptoms do not appear commonly until very near the physiological maturity of the plant (*9*), it was impossible to make accurate readings in these late-maturing segregates because low average temperature retarded development of near-wilt. Furthermore, secondary resistance derived from Penin may have caused some masking of susceptibility. In the other two crosses which deviated significantly from a 1:2:1 ratio, the deficiency was in the susceptible class. It is possible that classification in these was affected by retarded near-wilt development, although such was not so evident at the time as in the case of C127.

TABLE 2.—*Reactions of F₃ progenies on near-wilt soil near Waupun, Wis., in 1944; susceptible plants discarded in F₂*

Susceptible parent	Cross number or variety	Total progenies	Progenies in class indicated			Chi-square for goodness-of-fit to 1.2 ratio
			Resistant	Segregating	Susceptible	
		Number	*Number*	*Number*	*Number*	
Perfected Wales	C125	22	6	16	---- ----	0 14
	C129	25	9	16	----------	.01
	C132	21	6	15	----------	.05
	C135	10	3	7	----------	(¹)
	C138	5	1	4	----------	.03
	Perfected Wales	² 70	2	----------	68	----------
	Wisconsin Perfection	33	1	----------	32	----------
Merit	C126	23	3	18	³ 2	2. 63
	C130	13	4	7	³ 2	(¹)
	C133	20	6	14	----------	.01
	C136	15	2	10	³ 3	.84
	C139	16	7	8	³ 1	.68
	Merit	68	6	----------	62	----------
	Wisconsin Perfection	28	0	----------	28	----------
Penin	C127	29	8	19	³ 2	.04
	Wisconsin Perfection	69	1	----------	68	----------
Wisconsin Perfection	C128	37	6	31	----------	4. 14*
	C131	33	9	24	----------	.31
	C134	5	0	5	---- -----	1. 23
	C137	4	0	4	----------	.78
	C140	5	1	4	----------	.03
	Wisconsin Perfection	146	5	----------	141	----------

*Significant, 5 percent.
¹ Value for Chi-square less than 0.01.
² Individual plants tabulated in susceptible checks.
³ Probably from susceptible escapes in the F₂ progeny in 1943.

TABLE 3.—*Reactions of F₄ progenies from heterozygous F₂ plants on near-wilt soil near Waupun, Wis., in 1944*

Susceptible parent	Cross number or variety	F₂ plants	Total progenies	Progenies in class indicated			Chi-square for goodness-of-fit to 1:2:1 ratio
				Resistant	Segregating	Susceptible	
		Number	*Number*	*Number*	*Number*	*Number*	
Perfected Wales	C125	3	57	16	22	19	3. 28
	C129	3	68	20	32	16	.71
	C132	5	125	38	69	18	7 75*
	C135	2	44	17	19	8	4. 50
	C138	4	72	31	26	15	12 67**
	Perfected Wales		¹ 283	8	----------	275	----------
	Wisconsin Perfection		281	5	----------	276	----------
Merit	C126	2	44	11	23	10	.14
	C130	4	90	28	41	21	1. 80
	C133	6	117	23	60	34	2 15
	C136	4	75	22	40	13	2. 49
	C139	3	58	15	30	13	.21
	Merit		283	34	----------	249	----------
	Wisconsin Perfection		403	5	----------	398	----------
Penin	C127	11	228	94	94	40	32. 60**
	Penin		241	9	----------	232	----------
	Wisconsin Perfection		174	1	----------	173	----------
Wisconsin Perfection	C128	4	126	28	63	35	.78
	C131	6	90	24	44	22	.13
	C134	5	123	30	64	29	.22
	C137	4	92	28	45	19	1. 80
	C140	4	93	29	46	18	2. 61
	Wisconsin Perfection		627	37	----------	590	----------

*Significant, 5-percent; ** significant, 1-percent.
¹ Individual plants tabulated in susceptible checks.

The only F_1 which was tested on diseased soil was that of cross C141, tested in 1944. On July 24, the last date on which readings were made that season, all 26 plants of C141 were healthy while 60 plants of the resistant parent (738–49/I) on one side of the F_1 were healthy, and 50 plants of the suceptible parent (lot 23) on the other side had wilted and 4 were healthy. In this case, then, resistance was completely dominant in the F_1, as would be expected from the type of segregation in F_2.

It has been pointed out that, environment being favorable, the onset of the near-wilt disease in the field is delayed until shortly before the plant approaches physiological maturity. Since canning peas usually mature quite rapidly and since the plants dry out as the seeds become ripe, the normal period for the development of the disease is short. Moreover, environmental conditions have a marked effect on disease development. In 1945 the season was moist and cool throughout and very little near-wilt developed on the same plot where excellent results were obtained in 1943 and in 1944 with the exception of the late-maturing progenies.

Resistant selections from cross C140 (table 3) were crossed with several susceptible varieties and the F_1 was grown on near-wilt-free soil in the field in 1945. The F_2 progenies were planted on the Waupun near-wilt plot in 1946. Near-wilt developed abundantly, but severe drought and hot weather preceded appearance of symptoms and so hastened plant maturity that it was impossible to distinguish signs of near-wilt from those of premature ripening. An attempt was made to record the healthy and diseased plants in the F_2 progenies, although accurate classification was not possible. The results are presented in table 4. Twelve of 19 progenies did not show a good fit to a 3:1 ratio.

It became apparent, after field trials for four successive seasons on the Waupun plot that seasonal environment so influenced the expression of near-wilt that accuracy of disease readings might be impaired. In two seasons, 1943 and 1944, conditions were favorable for the full expression of near-wilt. In the following seasons readings were impaired in one case by premature maturation caused by excessive drought and heat, and in the other case by too low average temperature. In 1947, an attempt was made to intensify disease development by planting seed in heavily inoculated field plots at Madison, Wis. Concentrated inoculum of the near-wilt organism was applied in four ways: (1) Heavily infested soil in the row under the seed; (2) heavy liquid suspension of spores and mycelium in the row under the seed; (3) application of a suspension after (2) to the soil near the roots when plants were 6 inches high, and (4) when plants were 12 inches high. No diseased plants resulted even in susceptible progenies. Very few diseased plants developed in a susceptible variety on the Waupun plot the same year. The uncertainty of field analysis of progenies segregating for resistance thus became a decidedly limiting factor in this study and in a concomitant breeding program of developing near-wilt-resistant varieties. For this reason the possibilities of greenhouse testing were explored.

TABLE 4.—*Reaction of the F_2 progenies on near-wilt soil near Waupun, Wis., in 1946 and in greenhouse tests; and of F_3 progenies in greenhouse tests*

Cross	Parents	F_2 in field, Waupun, 1946			F_2 in greenhouse			F_2 in greenhouse		
		Resist-ant plants	Suscep-tible plants	Chi-square for goodness-of-fit to 3 . 1 ratio	Resist-ant plants	Suscep-tible plants	Chi-square for goodness-of-fit to 3 : 1 ratio	Resist-ant plants	Suscep-tible plants	Chi-square for goodness-of-fit to 5 : 3 ratio
		Num-ber	*Num-ber*		*Num-ber*	*Num-ber*		*Num-ber*	*Num-ber*	
C150	Alsweet × C140	143	85	17 69**				20	18	1 19
C151	737–1 × C140	259	148	27 43**	16	4	0. 07	72	52	.86
C152	534–12 × C140	459	300	84 64**	16	4	.07	43	22	.23
C156	741–33 × C140	387	75	18 47**				123	12	45. 94**
C157	532–6 × C140	145	50	.02	14	4	(¹)	50	27	.10
C158	733–7 × C140	211	61	.83	16	4	.07	50	31	(¹)
C159	739–22 × C140	243	54	7 00**				22	24	3. 62
C160	Lot 10 × C140	339	190	33 04**	21	2	2. 45	64	45	.51
C161	Lot 11 × C140	175	25	16. 01**				43	19	97
C162	Lot 12 × C140	56	19	(¹)						
C163	Lot 13 × C140	109	30	.69				30	16	.05
C164	Cannel King × C140	217	67	.23				39	13	2. 95
C165	Pride × C140	91	39	1. 48				34	18	.08
C166	Lot 23 × C140	126	60	4. 85*	22	3	1. 61	47	38	1. 59
C167	733–23 × C140	75	4	15. 70**				40	17	1. 12
C168	733–42 × C140	41	1	10 29**				9	9	.73
C169	Alderman × C140	683	115	47. 16**	96	32	(²)	16	9	(¹)
C171	Prince of Wales × C140	251	76	.45				47	16	3. 44
C174	744–12 × C140	339	139	4. 03*						
C178³	Loyalty × Delwiche Commando				52	17	(¹)			

*Significant 5-percent; **significant 1-percent.
¹ Value for Chi-square less than 0.01.
² Total of 2 trials (50 : 10 and 46 : 22) made 1 month apart.
³ Cross made in 1946.

GREENHOUSE TESTS

It was shown by Virgin and Walker (*9*) that near-wilt develops more slowly than wilt in the greenhouse over a range of soil temperatures. Schroeder and Walker (*4*) showed that as the temperatures of wilt-inoculated quartz sand increased, the disease increased up to about 28° C. and as it approached the optimum, varieties perfectly resistant to wilt in the field succumbed. In setting up greenhouse tests for near-wilt it became necessary to determine a set of controlled and readily reproducible conditions under which repeated tests could be made. The numerous tests made with Delwiche Commando and Wisconsin Perfection varieties to determine a standard technique will not be presented in detail. Soil as a substrate was found to be unreliable, and rapid disease development was not attained. Quartz sand inoculated by the method of Schroeder and Walker (*4*) did not give rapid or consistent results with near-wilt. Increase of temperature toward the optimum (28°) resulted in erratic disease development in Delwiche Commando. More consistent results were secured with plants grown in sand until 2 inches high, removed for inoculation, and replanted. Dipping the roots in concentrated inoculum did not give as rapid nor as consistent results as clipping the taproot uniformly to 1-inch length while the entire root system was immersed in the inoculum. This method gave the most satisfactory results when the sand temperature was kept at 21°.

FIGURE 2.—Peas grown in sterilized quartz sand at 21° C. and inoculated with *Fusarium oxysporum* f. *pisi* race 2. Odd-numbered rows, Delwiche Commando, even-numbered, Wisconsin Perfection. Rows 1 and 2, inoculated by dipping roots in spore suspension; rows 3, 4, 7 and 8, inoculated by clipping taproots to 1 inch with entire root system immersed in suspension; rows 5 and 6, uninoculated.

A comparison of the last two methods is illustrated in figure 2. The cut-root dip method was used for all subsequent greenhouse progeny tests.

Since the 1946 field trials with F_2 populations did not confirm those of 1943, remnants of F_2 seed were planted in the field plot in 1947 and seed of single F_2 plants was saved. Inasmuch as near-wilt did not appear in the 1947 plot; no elimination of susceptible plants occurred. Since limitation of space prohibited testing each individual 1947 plant progeny in the greenhouse, two seeds were taken from each F_2 plant and bulked to form an F_3 population representing each F_2 plant. If the two seeds selected in the heterozygous families were at random, then segregation based on a single-gene difference between parents would be expected in a ratio of 5 resistant to 3 susceptible individuals. Some F_2 seeds from a few of the crosses were still available, as were larger amounts from crosses C169 and C178. These lots were also tested by the greenhouse method.

The results of the greenhouse trials are presented in table 4. Seventeen families were tested in the F_2 at Waupun in 1946 and in the F_3 in the greenhouse. In the 1946 season, which was unfavorable for disease evaluation 11 families deviated significantly from the expected 3:1 segregation. Under the much more exacting greenhouse test, 16 of the families segregated close to the expected 5:3 ratio. The deficiency in susceptible segregates in 741–33 × C140 in both field and greenhouse has not been explained. In seven families tested in the F_2 in the field in 1946 and in the greenhouse, five deviated

significantly from the expected 3 to 1 in the field while all showed a good fit in the greenhouse.

The greenhouse results confirmed the field results of 1943 and 1944 in showing that crosses of susceptible varieties with a resistant parent which was extracted from one of the original crosses yielded confirming 3:1 segregation in the F_2 and 5:3 in the F_3. They also showed conclusively that a relatively simple greenhouse technique can be used to differentiate individuals in segregating progenies into discontinuous near-wilt-resistant and near-wilt-susceptible classes. Field tests for this purpose are often unreliable because of the short period of complete near-wilt expression and the great liability that unfavorable environment will preclude accurate differentiation.

DISCUSSION

There are several factors which complicate the separation of pea plants into resistant and susceptible classes on the basis of reaction to near-wilt. One of these is the presence of a secondary type of resistance in some varieties such as Rogers K and Horal. This type of resistance will often carry through when conditions for disease development are below optimum, and under favorable conditions will retard development of the disease as compared to that in varieties like Wisconsin Perfection which do not possess the secondary resistance.

With favorable conditions for disease development a small percentage of diseased plants appeared consistently in the resistant parent line. The symptoms, however, differed from those of near-wilt in that there was more cortical rotting of the roots and slower dying of the leaves in contrast to the rapid unilateral yellowing, wilting, and dying of typically diseased plants. On the other hand, in all field trials thus far there have always been occasional escapes in susceptible varieties under the most favorable conditions for near-wilt. Diseased plants in resistant progenies and escapes in susceptible varieties have been recognized in pure-line material. Undoubtedly, in combination with other factors they have affected the accuracy of classification of segregating progenies.

The most disturbing influence on accuracy of classification has been that of environmental factors. The most important of these factors has been cool weather which favored mild development of the disease and a large number of escapes. While the optimum for wilt in the field is 22° C., that for near-wilt is from 24° to 28° (9). Near-wilt develops more slowly than wilt when each are at their respective optimum. The delayed appearance of near-wilt is also influenced by the fact that varieties differ in the rate of disease development, the disease progressing more slowly in later-maturing varieties. The high temperature needed for optimum disease development and the association of the latter with approaching host maturity shortens the period during which symptoms are expressed. This is illustrated in figure 1, where it is to be noted that although all plants of the susceptible variety were dead, they had produced as many and as large pods as the resistant variety before they succumbed. The first symptoms in this case were noted during the first week in July and when the photograph was made on July 12 all susceptible plants were dead. A hot, dry season like that of 1946 may disturb classification of

plants as badly as a cool season because healthy plants mature and die so quickly after the appearance of near-wilt that it becomes impossible to distinguish those killed by drought from those killed by near-wilt.

The method of testing used in the greenhouse also has disadvantages. Besides the obvious limitation in amount of material that can be handled, resistance may break down somewhat with the treatment required to produce satisfactory elimination of susceptible plants. The difference between the resistant and susceptible classes was too small at 24° C. while at 21° it was usually sufficient for practical purposes (fig. 2). However, even at the latter temperature some variation in severity of disease occurred between runs, but the reasons are not understood. To compensate for this variation, classification of segregating material was always made in reference to known resistant and susceptible varieties grown in the same pan.

General observations and surveys of the canning crop in Wisconsin have shown variations in amount of near-wilt from season to season in those areas where it occurs in conformity with the variations recorded here. Although several recent seasons have been unfavorable for its development, near-wilt remains a major disease of the canning crop in Wisconsin and seasonal variation in weather may provide more nearly optimum conditions in future years. Since the resistance now available in line 738-49, unlike wilt resistance, does not occur naturally in any of the commercial varieties tested, desirable types with near-wilt resistance will have to be developed by hybridization. Since the high degree of resistance in the 738-49 line is shown to be controlled by a single dominant gene, greatest progress can probably be made with the application of the controlled greenhouse testing technique described herein for the screening of breeding materials for near-wilt resistance.

SUMMARY

A high type of resistance to near-wilt of garden pea, incited by *Fusarium oxysporum* f. *pisi* race 2, is described. This resistant character was apparently inherited from one of the Admiral selections used as parents of strain 732-66-1 in which resistance was discovered.

Evidence from the F_2, F_3, and F_4 generations of one set of crosses, and from the F_2 and F_3 generations of a second set of crosses involving resistant selections from the first group shows that the inheritance of resistance is dependent upon a single dominant gene.

Difficulties in testing for resistance to the disease under field conditions over a period of years are indicated. A method of testing with controlled environment and inoculation in the greenhouse is described.

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SOME ASPECTS OF THE LONGITUDINAL GROWTH OF BROMEGRASS FRUITS [1]

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INTRODUCTION

Although the growth of many plants and plant organs has been extensively studied, little research seems to have been directed toward the growth of grass fruits. This aspect of growth is important in view of the large economic value of the fruits of the Gramineae and the possible use of such information in agricultural practice.

In 1920 Harlan (4),[3] working on Hannchen barley, made a study of the growth of grass fruit from flowering to maturity. He found that most of the growth of the kernel was made in the first 3 days after pollination. The growth curve showed a sharp rise at first, a leveling, and then a small dip at the end. Half the growth in length was made from the second to the fourth day after fertilization, and in 7 days maximum length had been attained. After the peak was reached, there was a gradual falling off toward maturity.

Pope (8) reported that in barley kernels growth limitation is largely due to starch congestion. Such large amounts of starch are stored in the endosperm cells that mitotic division ceases. Deposition of starch in amounts sufficiently large to hinder cell division places a limit on the maximum size of the kernel.

Harlan and Pope (5), in a study of growth in immature barley kernels removed from the plant, noted that the endosperm and embryo grew at least 8 days after the heads had been harvested. The explanation offered for this was that food materials passed from the severed culm into the developing fruit. The fruits were almost 4 mm. long 2 days after pollination, 6 mm. long after 3 days, 8 mm. after 4 days, 8.8 mm. after 5 days, and by the end of the seventh day the plumule and radicle were visible.

Booth (*1*) reported that growth in length of the oat kernel was most rapid during the first 6 days after pollination.   It then became more gradual until the fifteenth day, when the maximum was reached.

MATERIALS AND METHODS

Fruits were collected during 1945 and 1946 from 50 mature plants of smooth bromegrass (*Bromus inermis* Leyss.) of the Achenbach strain growing in the Farm Crops nursery at East Lansing.   The plants used represented a fairly random sample of the hundreds of plants in the nursery.   Individual spikelets were tagged when the basal floret on each came into anthesis.   Tagging proceeded during most of the flowering period of the plots, and specimens were collected each day after the first day of tagging, thus "a 1-day-old fruit" was collected 24 hours after pollination.   It is highly probable, as pointed out by Nielsen (*7*), that fertilization takes place 15 to 18 hours after pollination.   Although the author (*6*) had previously shown that, under favorable conditions, the anthesis of florets in a bromegrass spikelet proceeds evenly, the basal floret opening first, followed the next day by the one above it, and so on apically until the sterile floret is reached, the variable climatic conditions of 1945 and 1946 made it unsafe to proceed on this basis.   Accordingly, only the basal floret or fruit was removed in each case.   As these were collected, the fruits were immersed in strong Navashin (Craf) solutions, as outlined by Sass (*9*).   Evacuation of the air in the fruits was followed by direct measurements with a millimeter rule placed under the 32-mm. objective of a dissecting microscope.   Fruits were measured from their base to their apex, omitting the protruding hairs.

PRESENTATION OF RESULTS

In 1945 measurements were made on a tentative basis and were carried only up to the fifteenth day after pollination.   The measurements for 191 fruits for this period are shown in table 1.   No 9-day-old fruits were obtained in this year.   The length of the 1-day-old fruits was approximately that of the ripe ovary, which is understandable when one considers that fertilization occurred 6 to 9 hours before gathering.

During the first 15 days the fruit grew from a length of 1.5 mm. to 9.5 mm.   Growth was slow at first, only 0.4 mm, or 5 percent, of the total growth occurring in the first 4 days (table 2).   From the fourth

TABLE 1.—*Average measurements of the length of developing bromegrass fruits in 1945*

Day No.	Fruits	Average length	Day No.	Fruits	Average length
	Number	*Mm.*		*Number*	*Mm*
1	16	1.5	9	0	---------
2	15	1.6	10	15	8.9
3	15	1.7	11	18	9.0
4	14	1.9	12	15	8.7
5	12	5.4	13	18	8.8
6	14	6.5	14	6	9.5
7	16	7.6	15	5	9.5
8	12	7.8			

TABLE 2.—*Periodic increases in length, by percentages, of bromegrass fruits in 1945*

Days	Increase in growth	Percent- age of total increase	Days	Increase in growth	Percent- age of total increase
	Mm.			*Mm.*	
1-4	0.4	5.0	10-15	.6	7.5
4-5	3.5	43.75			
5-10	3.5	43.75	Total	8.0	

to the fifth day, however, growth was very rapid, that is, 3.5 mm., or 43.75 percent, of the total gain in length. Similar growth was made from the fifth to the tenth day, but only 0.6 mm., or 7.5 percent, was made the last 5 days.

The upper curve of figure 1 shows the growth of the 191 fruits. This curve, based on the data in table 1, shows that early slow growth was followed by a period of greatly accelerated increment and then by a gradual slowing down.

In 1946, a more intensive study was conducted on 225 fruits. The measurements of these fruits are given in table 3. These were carried through to the twentieth day but some days are not represented. In most cases failure to collect was due to the fact that the desired fruits did not develop. Some inconsistencies in average length on certain days may be noted. These have been due to sampling errors or to fluctuations in climatic conditions, or both. The fruits grew from 1.6 mm. to 9.8 mm. and at maturity measured only 8.5 mm. The decrease in length at maturity may be explained as a dehydration phenomenon normally accompanying the final maturing of the fruit.

Table 4 reveals that, as in 1945, growth was slow at first. During the first 3 days the fruits grew 0.7 mm., or 8.5 percent, of their total length. From the third to the fifth day, they grew 6.5 mm., or 79.3 percent; from the fifth to the ninth day they grew only 1.0 mm., or 12.2 percent, while from the ninth to the twentieth day they showed a negative growth of −1.0 mm.

The lower curve of figure 1 demonstrates the progress in growth in 1946 fruits and shows the same general features exhibited by the 1945 curve. It is apparent that acceleration in the rate began earlier in 1946 than in 1945 and proceeded somewhat more rapidly during the early period. In comparing the two curves with the percentages in tables 2 and 4, it can be seen that in 1945, 48.75 percent of the growth

TABLE 3.—*Average measurements of the length of developing bromegrass fruits in 1946*

Day No.	Fruits	Average length	Day No.	Fruits	Average length
	Number	*Mm.*		*Number*	*Mm.*
1	10	1.6	11	8	8.4
2	1	1.7	12	4	8.7
3	5	2.3	13	0	
4	40	5.1	14	68	9.3
5	14	8.8	15	0	
6	15	9.6	16	17	9.5
7	11	9.6	17	0	
8	0		18	0	
9	13	9.8	19	0	
10	13	7.8	20	6	8.5

had occurred by the end of the fifth day. In 1946, by the end of the fifth day 87.8 percent of the growth had occurred.

Since weather conditions affect the rate of growth (2), a summary of the weather conditions prevailing in the East Lansing area in June and July of 1945 and 1946 is given (table 5). The data show that
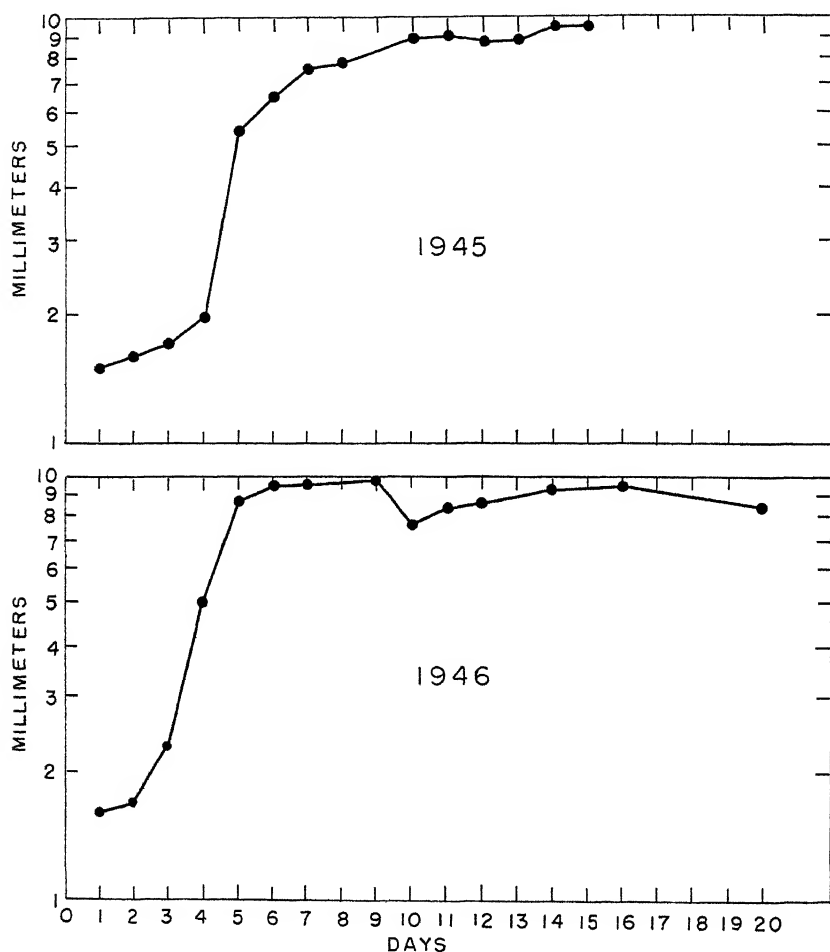
FIGURE 1.—Growth curves of bromegrass fruits in 1945 and 1946.

during these 2 months of fruit development, the temperature was higher in 1946 than in 1945, if one adds the accumulated excess for the 2 months in each case. There was also less deficiency in precipitation and a higher percentage of the possible sunshine in 1946 than in 1945. These differences in climatic conditions may account for the differences in the growth curves.

TABLE 4.—*Periodic increase in length, by percentages, of bromegrass fruits in 1946*

Days	Increase in growth	Percentage of total positive increase	Days	Increase in growth	Percentage of total positive increase
	mm.			*mm.*	
1-3	0. 7	8 5	9-20	−1. 0	−12. 2
3-5	6. 5	79 3			
5-9	1. 0	12. 2	Total	8 2	

TABLE 5 —*Summary of weather conditions in June and July of 1945 and 1946 at East Lansing, Mich*

Item	1945		1946	
	June	July	June	July
Accumulated excess (+) or deficiency (−) in mean temperature since first day of month_____9F..	+21	+8	+36	+2
Excess (+) or deficiency (−) in precipitation for the month as compared with normal rate_____ _____inches..	+ 19	−1 02	−1 03	−2 85
Accumulated excess (+) or deficiency (−) since Jan.1__inches..	− 27	−. 15	−. 08	− 09
Mean percentage of relative humidity:				
7 30 a. m_____	80	80	76	74
1:30 p. m_____	56	50	55	40
7:30 p. m_____	62	55	61	46
Total hours actual sunshine_____	265	336	268	386
Percent of possible hours sunshine_____	58	72	58	83

DISCUSSION OF RESULTS

The growth curves of bromegrass fruits in 1945 and 1946 follow closely the pattern shown for other fruits by Gustafson (*3*), for barley by Harlan (*4*), and for oats by Booth (*1*)—Both curves show the initial slow period, the tremendous increase in rate thereafter, followed by a slackening. In barley most of the fruit growth was made in the first 3 days, in oats the greatest length was attained by the sixth day, while in bromegrass most of the growth was made by the end of the fifth day. Different climatic conditions might, of course, produce a different result.

Some of the reasons usually given for the four distinct periods in the growth curve, slow growth, rapid growth, leveling-off growth, and negative growth, are: (1) an increase or decrease in cell multiplication; (2) interference with mitosis by starch accumulation in the endospermal cells; (3) the absorption or loss of water in the tissues of the fruit; and (4) genetic factors. An anatomical investigation now under way on the embryological development of the species will attempt to correlate the tissue development with the growth curve and may give some explanation for the periods.

SUMMARY

Bromegrass fruits were measured in 1945 and 1946 and growth curves were established for those 2 years under the prevailing climatic conditions. The growth curves agree closely with those of other fruits. In bromegrass most of the longitudinal growth in the fruit had occurred by the end of the fifth day.

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THE EFFECT OF SUNLIGHT ON THE ASCORBIC ACID CONTENT OF STRAWBERRIES [1]

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INTRODUCTION

Although it is generally realized that environmental factors may influence the nutritive value of fruits and vegetables, there has not been a great deal of research reported to show the extent to which climatic variants may exert their effect. The work reported in this paper is concerned with the relationship between sunlight and the ascorbic acid content of strawberries.

Reports in the literature on the ascorbic acid-sunlight relationship for strawberries are rather conflicting, although they are agreed that there is such a relationship. McCrory (*3*)[3] found that long-stemmed fruits were higher in ascorbic acid than those with shorter stems, and that fruit exposed to sun was higher than that shaded by leaves. He also reported that fruit harvested on clear days had a higher ascorbic acid content than those harvested on cloudy days. However, these observations were not supported with data. Hansen and Waldo (*2*) studied the problem by shading entire plants, or the berries alone, with muslin bags and concluded that under the former condition a much lower amount of ascorbic acid was synthesized. The effect of shading the berries alone was not so great. However, the statistical significance of these observations could not be determined by the data presented. Ezell and associates (*1*) found that when entire plants or only the berries were shaded, less ascorbic acid was accumulated than with full illumination. These workers used one and four thicknesses of cheesecloth for the plot-shading experiments and brown manila bags for the shading of the fruit alone. They also related climatological data published by the United States Weather Bureau to the seasonal fluctuations of the strawberry ascorbic acid and deduced that the amount of sunlight for approximately 1 week prior to harvest is operative on the ascorbic acid production.

In a survey of the ascorbic acid content of 81 varieties of strawberries in 1946 Robinson *et al.* (*4*) found a statistically significant difference of 15 percent in the average ascorbic acid content (fresh-weight basis)

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of the fruit grown at two different locations. Strawberries grown on Dunkirk fine sandy loam 5 miles north of Geneva contained more ascorbic acid than those grown on Dunkirk silty clay loam at Geneva.

EXPERIMENTAL PROCEDURE

Two types of experiments were undertaken; one involved a comparison of the ascorbic acid content at the two locations mentioned above, and the other involved a study of the influence of shading upon the ascorbic acid content of strawberries. One light recorder was installed at each of the two locations, i. e., at Geneva and at the farm 5 miles north of Geneva, in order to obtain a comparison of the amount of illumination at the two localities. Nine varieties of strawberries grown at each location were compared twice during the season.

The integrating light recorders used are a modification of the instrument described by Sprague and Williams (*6*). The same principles of operation are involved, but the details of the electrical circuit and recorder are somewhat different. The phototube housing is composed of a brass case on top of which is mounted an opal glass globe. The top of the brass case has an aperture containing 1-inch-square glass filters and a ground-glass plate. The filters are Corning Nos. 3962 and 3850, which in combination transmit a very broad band of light, principally in the visible portion of the spectrum with a maximum at about 5,000 Å. Some ultraviolet light longer than a wave length of 3,700 Å. is transmitted and there is a small amount of transmission beyond 7,500 Å. (up to 10,000 Å). This combination, in conjunction with RCA 922 phototube, gives two peaks in the maximum sensitivity curve. One maximum is at about 4,000 Å. and the other is at about 6,000 Å.

The shading experiments were all made at Geneva. The effect of shading only the fruit was studied by placing white parchment paper bags over them a few days after the blossoming stage. The paper was found to reduce the illumination to 52 percent of that of full sunlight, as measured by the integrating light recorder. One plot of berries was entirely covered by a cheesecloth cubical (12′ x 12′ x 12′) that enclosed a light recorder as well. The amount of light reaching the strawberries thus shaded was 43 percent of that reaching the fully exposed plants. The variety Premier (Howard 17) was used for all of the shading tests.

In all cases, the fruit was picked between 8 and 9 o'clock in the morning and analyses were completed the same day. Ascorbic acid analyses were made on individual berries by the xylene-extraction modification of the indophenol method (*5*). Although ascorbic acid analyses were made on individual fruits each day, only the averages for each day with the standard errors are reported here. Statistical analyses were made by the paired comparison method of Student (*7*, *8*). On the same day, the averages of each group in the shading test, and each variety in the soil test, were used for the pairing.

EXPERIMENTAL RESULTS

The fruits ripened in the cheesecloth cubical were invariably lower in ascorbic acid content than those ripened with either the leaves or the whole plants exposed to full sunlight (table 1). Each of these

comparisons was highly significant (odds greater than 4,999 and 9,999 to 1, respectively). The comparison between the fruit ripened in complete sunlight and that ripened with only the fruit shaded showed no significant difference. The data are presented on the fresh weight basis so that they can be more readily compared with other data in the literature. Calculation of the data for the shading experiments on either the fresh or the dry weight basis made no difference in the conclusions drawn from these tests.

The relation between the day to day variations in ascorbic acid and the amount of sunlight is shown in figure 1, where the ascorbic acid content of fruits from fully exposed plants (table 1) are compared with the daily sunlight records. The sunlight data are presented in graphical form, with the sunniest day, June 20, represented as 100 percent. The ascorbic acid level in the fruit corresponded fairly well with the relative intensity of the sunlight on the fifth or sixth day prior to ripening. The date of harvest is plotted 5 days ahead of the sunlight record in the figure to emphasize this relationship.
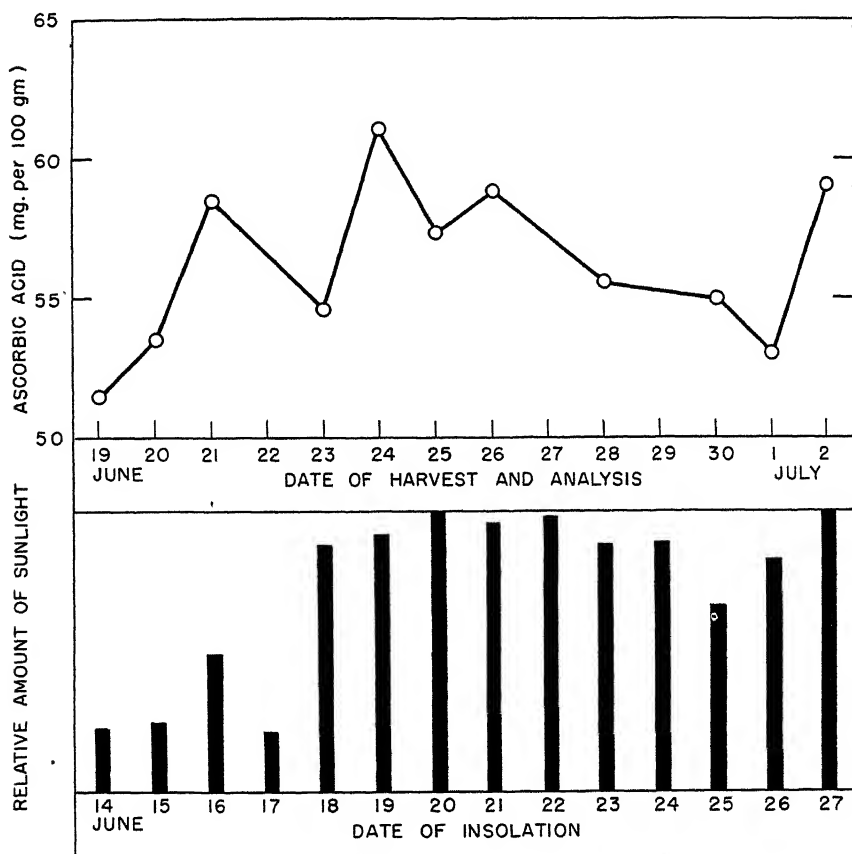
FIGURE 1.—Relation between amount of sunlight and ascorbic acid content of strawberries grown at Geneva, N. Y., 1947.

TABLE 1.—*Effect of shading on the ascorbic acid content of strawberries* [1]

Date, 1947	Full exposure to sun light		Fruit shaded, leaves exposed		Leaves and fruit shaded	
	Berries	Ascorbic acid	Berries	Ascorbic acid	Berries	Ascorbic acid
	Number	*Mg. per 100 gm.*	*Number*	*Mg. per 100 gm.*	*Number*	*Mg. per 100 mg.*
June 20	40	53 59±1 21	7	46.90±1 39	--------	------------
June 21	10	58 55±1.74	10	47 81±1.50	3	40 32±1 95
June 23	25	54 64±1 79	25	56 95±2 19	8	42 46±3.19
June 24	10	61 06±3 56	10	58. 12±1 45	--------	------------
June 25	10	57 41±3.26	10	65 56±3.66	10	34.35±1.64
June 26	12	58.66±3 28	12	60 81±3.63	12	40 95±2.47
June 28	8	55.78±3.41	8	49 04±1.63	8	40.27±1 66
June 30	11	55 22±2 06	11	53.70±2.74	12	32.28±1.18
July 1	12	53 08±2 44	12	53 40±3.19	12	29.82±1.83
July 2	15	59.27±2.09	15	50.46±1 98	15	30 42±2.06
Average	--------	56 72	--------	54.28	--------	36.36

[1] Data expressed on fresh weight basis, variety, Premier (Howard 17).

The comparison between the two locations mentioned above, in which nine varieties of strawberries were used, again gave significant differences in ascorbic acid content on the fresh weight basis (table 2). However, the difference in amount of sunlight was insignificant. There was 4.3 percent less sunlight at the location where the higher ascorbic acid content was found. Examination of the dry weight data showed that the berries grown on the sandy soil had a higher total solids content. Calculation of the ascorbic acid data on the dry weight basis eliminated the apparent differences due to location

DISCUSSION

It seems apparent that the leaves must be responsible for the synthesis of the ascorbic acid or its immediate precursors, since the fruit that was fully exposed to the sunlight was not significantly higher in ascorbic acid than the shaded fruit, and only when the whole plant was shaded did the ascorbic acid level fall greatly. These data confirm those of Hansen and Waldo (*2*) in this respect, although these investigators, as well as Ezell et al. (*1*), found more ascorbic acid in the fruit when the whole plant was exposed than when only the berries were shaded. It is possible that the difference in type or degree of shading may explain this disagreement. Hansen and Waldo used unbleached muslin bags, while Ezell and coworkers used brown manila paper bags. Since white parchment paper bags were used in the studies reported here more light probably reached the fruit and there may have been a temperature difference as well.

The 5- to 6-day lag in response of the ascorbic acid level of the ripe fruit to the amount of insolation would indicate that the synthesis of either ascorbic acid or its precursors is stimulated by sunlight before the table-ripe stage is reached. Possibly the lag period represents the time from cessation of translocation from the leaves to the fruit, or it may be the time necessary for the precursor, formation of which is stimulated by sunlight, to be transformed into ascorbic acid. The latter hypothesis receives support from the observation of Ezell and his coworkers that the ascorbic acid of strawberries that are picked

Table 2.—Effect of different locations on the ascorbic acid content of strawberries

Variety	Ascorbic acid			
	Fresh weight basis		Dry weight basis	
	Dunkirk fine sandy loam	Dunkirk silty clay loam	Dunkirk fine sandy loam	Dunkirk silty clay loam
	Mg. per 100 gm	*Mg. per 100 gm.*	*Mg. per 100 gm.*	*Mg. per 100 gm.*
Sparkle	80.4	61 0	616	522
Fairfax	82 0	82 3	628	640
Catskill	88 1	69.8	708	694
Dresden	85 9	77 3	792	740
24248	88 5	64 5	718	556
25460	97.8	79.0	792	762
28340	52.9	55.0	500	530
28597	76 0	64.1	530	569
USDA 3414	77 2	74 7	558	599
			649	
Average	81.0	69 7		623
Z value for comparison of difference	1.21		0.40	
Odds that difference is significant	200 1		5. 9·1*	

*Not significant.

before complete ripening continues to increase after separation from the plant.

That soil type may influence the composition of the strawberry fruit is demonstrated in the comparison of the fruit grown at the two locations. The difference noted in the ascorbic acid content of the strawberries was caused by a difference in the relative amounts of solids rather than differences in the amount of sunlight. The difference between the ascorbic acid content of the strawberries grown at the two locations when expressed on a fresh-weight basis disappears when these same results are expressed on a dry-weight basis. This indicates that other comparisons based solely upon a fresh weight basis may or may not be valid. There may or may not be a real difference in the amount of ascorbic acid which has been produced. Instead, there may simply be the same amount of ascorbic acid produced at each location, and the difference noted may have been caused by a difference in the water content of the strawberries, the silty clay loam having relatively more juicy berries than the plants on fine sandy loam.

The data presented in this paper confirm the observation of several other investigators that sunny days are essential for obtaining the maximum amount of ascorbic acid in strawberries. It is therefore to be expected that great variations in ascorbic acid content will occur in strawberries grown in different seasons and in different geographical locations.

SUMMARY

The synthesis of ascorbic acid in strawberries is stimulated by exposure to sunlight. With a 57-percent decrease in the amount of illumination during development and ripening, the ascorbic acid content averaged 36 percent lower. The data in this paper indicate that the degree of illumination of the leaves is the most important factor in this relationship.

The amount of ascorbic acid in the ripe fruit appears to correspond to the amount of illumination on the fifth or sixth day prior to harvest.

Differences in ascorbic acid content of strawberries on the fresh-weight basis occurred at different locations with the same amount of llumination. However, if the ascorbic acid is expressed on a dry-weight basis the differences due to environmental factors other than sunlight were insignificant.

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THE INFLUENCE OF DRYING ON SOIL BUFFERING IN RELATION TO AGGREGATION AND OTHER FACTORS[1]

By R. M. SMITH, *project supervisor, Soil Conservation Service, United States Department of Agriculture,* and D. R. BROWNING, *assistant in agronomy, West Virginia Agricultural Experiment Station*

INTRODUCTION

Acidity, buffering, base exchange, and exchange capacity are obviously all closely interrelated, and are recognized as some of the most important fundamental properties of soils. A great deal of work has been done to obtain a better understanding of these properties and much progress has been made. Since the work of Bradfield (5) [2] it has gradually become recognized that complex aluminosilicate minerals, or clay minerals, are one of the main sources of soil acidity and buffering as well as of physical activity in soils. He and others (3, 20) have shown that much of the behavior of these purified clay minerals is like that of weak acids. The other major source of reserve acidity and buffering of soils is recognized as organic in origin (3, 4, 21). The buffering powers of organic compounds are often so great that a small percentage of organic matter dominates the entire exchange and buffering properties of many soils (21, 32).

If the complex organic materials were omitted it would seem that comparatively simple expressions might well be worked out for acidity, buffering, and exchange capacity. · But after a number of years of work by many investigators there is still no widespread agreement as to the fundamental definition of exchange capacity and no absolute method for making buffer measurements that are directly applicable in the field.

As a practical procedure for determining a standard relative exchange capacity value the neutral ammonium acetate method is probably the most widely used and the most useful (31). However, it is recognized that exchange capacity values obtained by this or by other standard laboratory methods cannot be used as an absolute measure of the lime or base content at pH 7 in the field (20, 22, 24, 25, 28). Moreover, Bradfield (6) has shown that soils and clays have a strong buffering capacity above pH 7, and he has suggested that a more satisfactory, practical end point for exchange capacity is the equilibrium point with calcium carbonate in an atmosphere with a CO_2

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[2] Italic numbers in parentheses refer to Literature Cited, p. 282.

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concentration of the air. This end point would be at a pH value of about 8.2.

A different end point has been used by Baver (*3*) in carrying through the analogy between clays and weak acids. His use of the flex points of soil-base buffer curves gives end points at variable pH values. With pure clay systems the flex points are often clearly evident, but with soils these flex points may be indistinct. Further, the simple weak acid concept does not explain the strong buffering above the flex point which is typical of many soils. Another complication in this explanation as well as in other approaches to soil buffering is the difference associated with the neutralizing ion. There is a wide difference between such buffer curves with sodium and lithium on the one hand and calcium and barium on the other, so that buffering can be expressed only by specifying the ion involved.

In practical soil liming studies it has been shown that factors of from 1.5 to as high as 3.0 must be used to convert laboratory buffer values into field lime needs, even when mixing is complete and all the lime is fine and readily available (*25*, *28*). Kappen (*14*) has noted a similar discrepancy between laboratory buffering and field behavior, but has attempted to dismiss it as probably resulting from incomplete contact in the field.

The literature on base exchange contains many indications of some sort of build-up of exchange capacity (*8*). Pierre and Scarseth (*24*) considered that $Ba(OH)_2$ treatment probably built additional exchange, and they rejected the preliminary treatment with $Ba(OH)_2$ for base exchange capacity determinations as recommended by Kelley (*15*) on this basis. They found that for limed soils the results were essentially the same with $Ba(OH)_2$ pretreatment as with the direct $BaAc-NH_4Cl$ method, which they interpreted as indicating that liming, like treating a soil with an alkaline solution, results in a "build-up" of additional exchange complex. Kelley (*15*) considered that the $Ba(OH)_2$ treatment merely resulted in displacement of all of the potentially exchangeable $H+$ and he did not consider this to constitute a build-up of exchange. There are other references to the difficulty of replacing the $H+$ from exchange material. Kappen (*14*) discusses this at some length, and Magistad (*18*) has emphasized the extreme slowness of replacement of $H+$ from exchange material compared to the replacement of metallic ions, in conjunction with his argument that alkaline treatments build more exchange instead of merely releasing more $H+$ as indicated by Kappen.

Field data of Robinson (*26*) show that for a particular soil type there may be a significant positive relation between the pH and the exchange capacity, suggesting some influence of bases upon the exchange capacity. For samples of Hagerstown silty clay loam having little variation in organic matter content his data show a rather close relation between pH and exchange capacity by the neutral ammonium acetate method. With Dekalb silt loam the pH-base exchange capacity relation is also distinct, but in this case organic matter variations may account for a part of the correlation.

Experimental plot results reported by Salter and Schollenberger (*29*) suggest that liming may have caused an increase in the exchange capacity on 32-year-old plots. Without manure, unlimed plots show an exchange capacity of 8.01 m. e. as compared to 9.87 m. e. for limed

plots; and with manure the values are for unlimed, 8.89 and for limed, 10.51 m. e.

Drying has been considered by a number of investigators (*1, 9, 10, 11, 12, 13, 17, 27, 28*) as one of the factors that influence acidity. Most reports have indicated some decrease in pH due to drying, but some have shown an increase with certain soils. Coles and Morison (*10*) have studied the effect of drying upon acidity in considerable detail and their results are in general agreement with others reporting pH decreases. They found a considerable lowering of the pH of all mineral soils by drying at 98° C., the greatest effect occurring on the first day, but some additional lowering being evident for several days. Peat showed a slower change downward which extended out over a longer period. Coles and Morison concluded that these pH changes were reversible for mineral soils, nonreversible for organic materials. This conclusion is not entirely substantiated by their data, for the mineral soils generally failed to reach the original pH value even after a period of 57 days of soaking. In fact, there was no clear trend toward a return after about 2 weeks. But, at least, the pH change was to a considerable extent reversible with all mineral soils. Their results also showed the elimination of the drying effect by preleaching with HCl and the return on treatment with a base. This seemed to indicate that the effect of drying was due to some reaction of the exchangeable bases. However, the pH values obtained by leaching with HCl and washing with water were well below 3.0 in most cases, which might lead to some question as to whether all the excess HCl had been removed. If not all removed, then the heating might be expected to drive off some HCl and thus prevent a lowering or even cause an increase in pH. Actually, a slight increase was obtained in some cases, particularly with two samples treated with H_2O_2 as well as with HCl. The increase was more than 0.2, starting with values below pH 2.6. Exchangeable bases were decreased by drying; water-soluble Ca and P were increased.

The object of the present paper is to report data obtained from drying soils before determining the pH, especially in connection with measurements of buffering capacity; and to attempt to show how the influence of drying may help to clarify some of the indicated inter-relations of pH, buffering, base exchange, and exchange capacity.

METHODS

All pH values were obtained with a glass electrode pH meter. A soil-water ratio of 1 : 2½ was used in some cases; other values were obtained on soil pastes, as indicated in the tables. Drying was accomplished in an oven at 110° C. unless otherwise indicated. Total exchangeable bases and exchange capacity were determined by the neutral, normal ammonium acetate method (*31*). Organic matter values are by dichromate oxidation (*7*).

Certain details of technique will be explained under specific headings in the results.

SAMPLES

A variety of representative West Virginia soils and subsoils have been studied. These include normal surface soils and subsoils of the gray-brown podzolic group. Clay subsoils represented are of acid

shale, limestone, and alluvial origin. Silty and fine sandy soils are derived from acid shales and fine sandstones. A sample designated as "muck" was obtained under "half-bog" conditions in a high valley draining acid shale and sandstone upland.

Most of the soils in the study are known from petrographic observation to be rich in hydrous micas. Clays of the 2:1 type are probably dominant in most samples. Base exchange studies show that the 0.002-mm. clay fraction ordinarily has an exchange capacity of about 30 m. e. per 100 gm. by the ammonium acetate method (*32*). One electrodialyzed colloidal clay, from Hagerstown subsoil, has been included in the study. Its exchange capacity is about 60 m. e. per 100 gm. One sample of commercial kaolinite and one of Wyoming bentonite are also included.

RESULTS

INFLUENCE OF DRYING ON THE PH OF VARIOUS SOILS AND CLAYS

Under West Virginia conditions the highest soil pH values are invariably obtained on moist samples. In a few cases there is no significant difference due to drying, but in most cases drying causes a marked decrease. Typical values are shown in table 1. A variety

TABLE 1.—*The influence of drying upon the pH of different soil and clay samples*

Soil No.	Description	Organic matter	pH of samples after soaking 3 hours—starting with condition indicated		
			Field-moist	Air-dry	Oven-dry
1	Gilpin silt loam (gray-brown silt loam, upland surface soil)	*Percent* 3.4	4.9	4.75	4.2
2	Dekalb silt loam	2.9	5.7	5.3	4.9
3	Gilpin silt loam	4.3		5.1	4.4
4	Holston sandy loam terrace	1.3	4.8	4.4	4.15
5	Monongahela silt loam	3.5	5.75	5.3	4.9
6 (a)	Holston loam subsoil	.2	5.1	4.85	4.5
6 (b)	Leached with HCl, washed with H₂O			3.65	3.25
7 (a)	Zoar clay subsoil	.3	5.3	4.95	4.0
7 (b)	Leached with HCl, washed with H₂O			3.7	3.4
8	Blago silt loam	12.4	4.9	4.5	3.9
9	Sandy podzol B horizon	3.3		4.4	4.0
10	Silty subsoil	1.4		4.7	3.8
11	Woodland mull	11.8		5.4	4.7
12	Electrodialyzed collodial clay			3.6	3.2
13	Commercial kaolinite			6.8	6.0
14	Sand and bentonite mixture leached with HCl and washed with H₂O			4.6	3.8
15	Same, but leached separately			3.7	3.2

of soils and subsoils are represented as well as soils leached free of all bases, a sample of commercial kaolinite, one of bentonite, and one of electrodialyzed H+clay.

INFLUENCE OF DRYING ON SOIL BUFFERING

When increments of base are added in solution to soil suspensions and allowed to stand with occasional shaking, pH results as shown in table 2 are obtained. The results are seldom completely stable with time, but after about 2 days a fairly satisfactory equilibrium is reached with most soils. A similar equilibrium time has been used by others (*19, 20, 25, 30*).

TABLE 2.—*Influence of time on the pH of soil suspensions in contact with a base*

Soil	M. E. of base	pH of soil suspension after—					
		1 day	2 days	3 days	5 days	8 days	17 days
1	3.6 Ba	5.55	5 6		5.45	5.45	
	None	5 1	5.1	5.0			
3	3.6 Ba	6.3	6 0	6.2			
	5.2 Ba	7.3	6 7	6.7			
5	3.6 Ba	6.6	6.45		6.45	6.5	
6	3.6 Ba	8.1	7.5		7.6	7 1	
	None		4.9			4.8	4.8
7	1.9 Na		6.0		5.65	5.65	
	3.6 Ba	5.7	5.55		5.50	5.55	
	3.8 Na		6.6		6.0	6.15	

It the samples are then dried at 105° C. and redispersed in water the pH values are all lower than they were before drying. Table 3 shows some typical values. This table and figures 1 and 2 show that there is usually a greater decrease in pH after additions of base than before.

TABLE 3.—*The effect of drying upon soil pH following the addition of various increments of base*

Soil	M. E. and kind of base added	pH of samples after soaking 2 days in contact with base	Immediate pH of samples redispersed in water after oven drying following the 2-day contact with base	Difference
1	0	4.75	4.2	0.55
	2.0 Ba	5.25	4.5	.75
	3.7 Ba	5.6	4.8	.8
	5.6 Ba	6.9	5.1	1.8
2	0	5.3	4.9	.4
	2.0 Ba	6.4	5.4	1.0
	3.7 Ba	6 9	5.9	1 0
	5.6 Ba	7.05	6.6	.55
4	0	4.75	4.25	.5
	2.0 Ba	5 75	4.70	1 05
5	0	5.3	4.9	.4
	2.0 Ba	6 1	5.5	ᵗ 6ᵗ
	3.7 Ba	6 65	5.9	.75
	5.6 Ba	7.05	6 0	1 05
6 (a)	0	5.0	4.4	.6
	2.7 Ba	6.2	5.05	1.15
	5.2 Ba	6.75	6.0	.75
	7.8 Ba	7.3	6.75	.55
	0	5.05	4.4	.65
	2.4 Na	6.4	5.4	1.0
	4.9 Na	7.05	6.0	1.05
	7.4 Na	7 7	6.6	1.1
6 (b)	HCl leached and washed with H₂O	3.65	3 25	.40
7 (a)	0	5.0	4.0	1.0
	1.8 Ba	5.4	4.3	1.1
	3.6 Ba	5.75	4.8	.95
	7.2 Ba	7.2	5.95	1.25
	12.7 Ba	8.3	7.2	1.1
	0	4.95	4.0	.95
	2.8 Na	6.0	4.4	1.6
	3.9 Na	6.65	5.05	1.6
	4.9 Na	6.9	5.25	1.65
	5.8 Na	7.3	5.5	1.8
	7.8 Na	7.95	5.95	2.0
	9.7 Na	8.5	6.7	1.8
	11.7 Na	8.95	7.2	1.75
7 (b)	Leached free of base with HCl and washed with H₂O	3.7	3.4	.3
8	0	4.5	3.9	.60
	3.6 Ba	5.05	4.05	1.0
10	0	4.7	3.8	.9
	4.2 Ba	6.7	5.5	1.2
	6.9 Ba	7.6	6.95	.65
	7.0 Ca	7.6	7.0	.6

TABLE 3.—*The effect of drying upon soil pH following the addition of various increments of base*—Continued

Soil	M. E. and kind of base added	pH of samples after soaking 2 days in contact with base	Immediate pH of samples redispersed in water after oven drying following the 2-day contact with base	Difference
11	0	5.4	4.7	.7
	4.7 Ba	5.65	4.8	.85
	19.8 Ba	6.3	5.1	1.2
	25.0 Ba	7.0	5.4	1.8
12	0	3.55	3.35	.2
	8.2 Ca	4.05	3.45	.6
	16.4 Ca	4.35	3.5	.85
	24.4 Ca	4.65	3.75	.9
	33.0 Ca	4.9	4.2	.7
	40.8 Ca	5.45	4.9	.55
	49.2 Ca	6.1	5.3	.8
	57.2 Ca	6.8	6.2	.6
	65.8 Ca	7.55	6.75	.8
	74.2 Ca	8.35	7.05	1.3

This means that there is an increased degree of buffering following drying. It is also evident that the drying has drawn the soil material together into larger and more definite aggregates than in the normal or moist state. This effect is, of course, most evident when well-dispersed colloidal materials are dried.

THE REVERSIBILITY OF pH CHANGES DUE TO DRYING

A number of replicate flasks of soil and base were dried and redispersed as indicated in the previous section. pH values were then determined on separate flasks in duplicate after different time intervals of soaking. The results, presented in table 4, show some tendency for the pH to increase in all cases, but it seems evident that the samples which have returned most quickly to about the same or to higher values than were obtained before drying are samples with no base added. In fact, all soils without base seem to have returned to essentially the original pH or higher in the time allowed, except the "muck." This is further illustrated by figures 1 and 2, which show a more complete recovery without extra base than with base.

It appears in table 4 that the various soils with base added may have reached a relatively stable pH level during the time required for the samples without base to return to the original values or higher. However, to compare the effect of much longer periods of soaking, both with dried and undried samples, several samples of 300 gm. each were placed in beakers and base solutions were added. After stirring at intervals for 2 days the pH was determined and each sample was divided into three parts. One series was then oven-dried for 2 days; a second series was kept moist continuously; and a third series was allowed to dry in the air with an occasional rewetting. pH values were then determined at intervals on small portions of soil paste removed from each beaker. The results (table 5) show that after sufficient continuous soaking time there was only a small difference in pH whether the soil was originally oven-dried, continuously wet, or alternately wet and air-dried during the entire period.
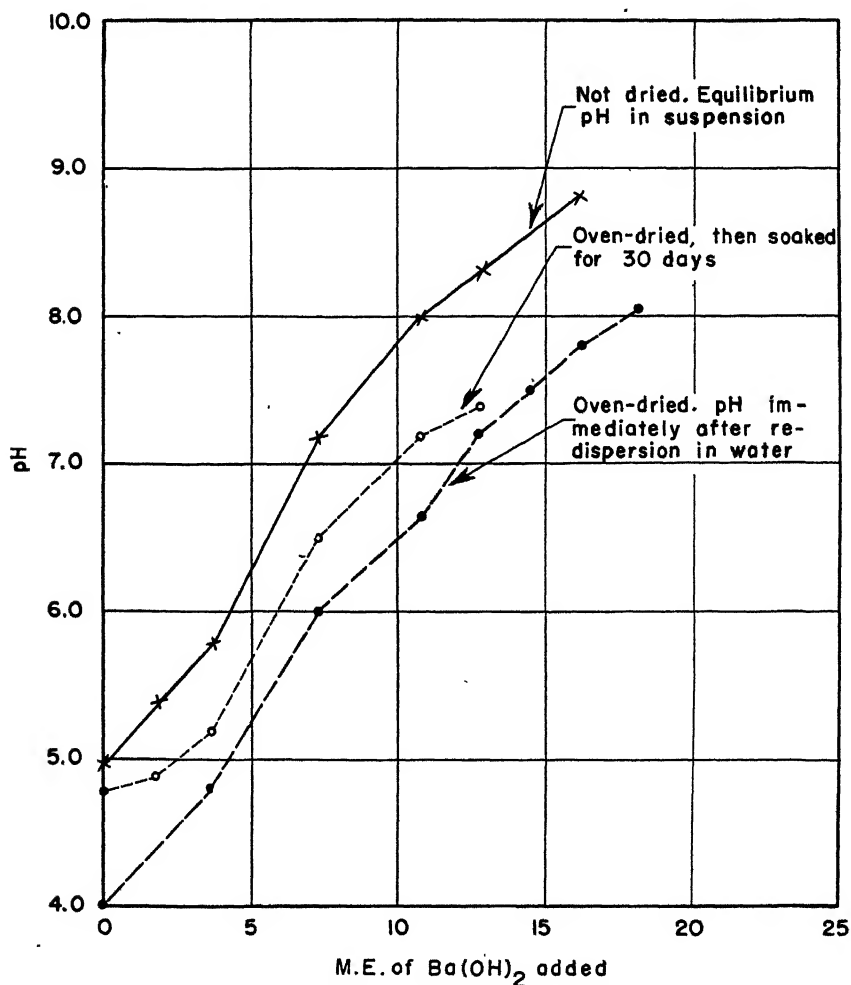
FIGURE 1.—Buffer curves of Zoar subsoil (soil No. 7a) showing the typical influence of drying and recovery after soaking. Note that the pH differences caused by drying are greater with base added than without and that the recovery by soaking is less complete.
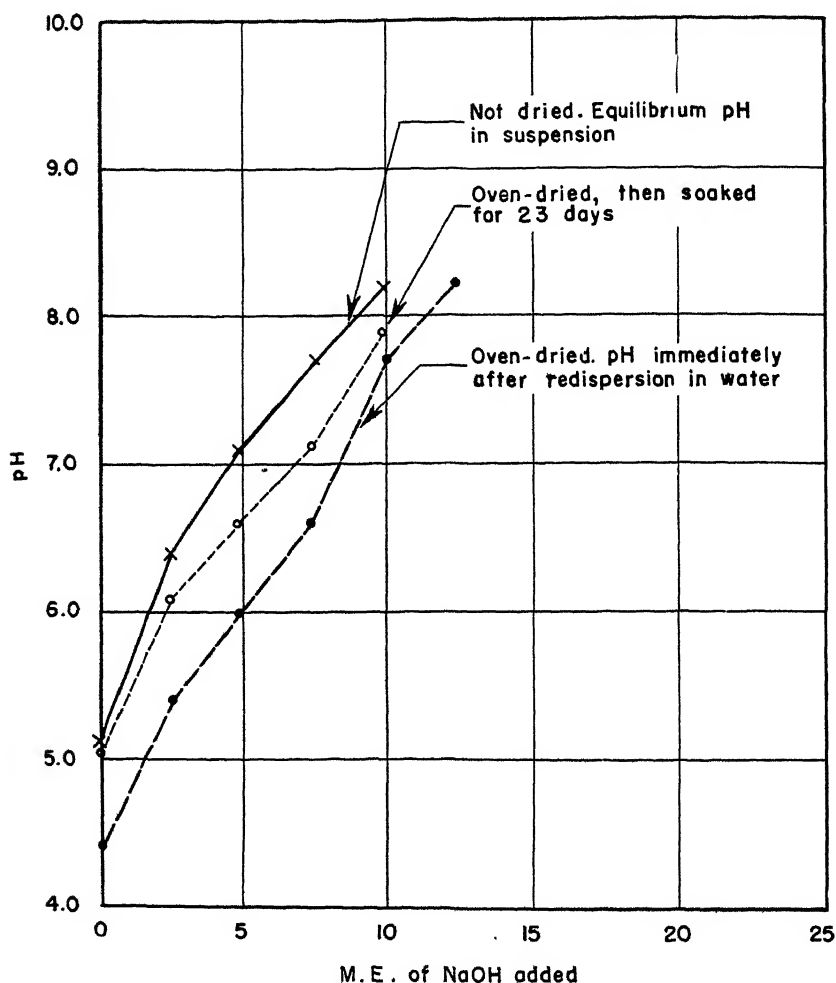
FIGURE 2.—Buffer curves of a Holston subsoil (soil No. 6a) showing the typical influence of drying and recovery after soaking. Note that the pH differences caused by drying are greater with base added than without and that the recovery by soaking is less complete.

TABLE 4.—*Reversibility of pH changes due to drying at 110° C.*

Dried, then soaked for time indicated	M. E. and kind of base per 100 gm. of soil		
	0	3.6 Ba	5.6 Ba
Soil 1·			
3 hours	4.2	4.8	5.1
6 days	4.4	5.4	
8 days	4.5	5.15	5.35
10 days	4.4	4.95	5.5
15 days	4 55	5.15	
22 days	4.75	5.25	5.55
Original value, undried	4.8	5.6	6.95
Soil 2			
3 hours	4.9	5.9	
1 day			6.6
6 days	5 3	6.7	
8 days	5 4	6.5	
9 days			6.9
10 days	5 3	6.4	
15 days	5 5	6.5	
22 days	5.6	6.45	6 75
Original value, undried	5.3	6.9	7.1
Soil 5.			
3 hours	4.9	5.9	5.95
2 days			5.95
4 days			6.6
6 days	5 6	6 6	
8 days	5.55	6 35	
10 days		6.2	6.85
15 days		6.15	6.7
22 days	5.4	6.15	6.7
Original value, undried	5.2	6.65	7.05
Soil 8 (muck):			
3 hours	3.7	4.05	
6 days	3.9	4.65	
8 days	3.90	4.45	
10 days	3.95		
15 days	4.1	4 5	
22 days	4.3	4 7	
31 days	4.35	4.65	
Original value, undried	4.9	5.05	

	0	2.7 Ba	4.9 Ba	7.9 Ba	10.4 Ba
Soil 3:					
5 minutes	4.4	5.1	6.0	6 75	7 5
20 days	4.9	5 6	6.5	6.95	7.1
Original value, undried	5.0	6.2	6.75	7.3	7.8

	0	1.8 Ba	3.6 Ba
Soil 6:			
3 hours	4.65	5.0	6.2
6 days	4.75	5.9	6.85
10 days	4.85	5.8	6.85
14 days	4.85	5.9	6.85
21 days	5.0		
30 days	4.95	5.6	7.0
Original moist value	5.1	6.95	7.65

	0	1.8 Ba	3.6 Ba	7.2 Ba	12.7 Ba
Soil 7:					
5 minutes	4.0	4.35	4.8	5.9	7.1
3 hours	4.3	4.50	5 0	6.3	
6 days	4.3	4.55	5.15	6.5	7.4
10 days	4.5	4 75	5.10	6 6	7.5
14 days	4.5	4.85	5.10	6.35	7.3
21 days	4.5	4.65	5.10	6 5	7.4
30 days	4.75	4.90	5.15	6.5	7.4
Original moist value	4.9	5.4	5.75	7 2	8.3

TABLE 4.—*Reversibility of pH changes due to drying at 110° C.*—Continued

Dried, then soaked for time indicated	M. E. and kind af base per 100 gm. of soil.				
	0	3.6 Ba	7.2 Ba	10.8 Ba	12.6 Ba
Soil 7:					
5 minutes or less	4.0	4.8	5.95	6.60	7.20
2 days	4.4	4.95	6.27	6.85	7.40
5 days	4.75	5.20	6.3	6.75	7.1
13 days	4.5	4.90	6.1	6.6	7.0
21 days	4.65	5.05	6.25	6.7	7.05
35 days		4.8	6.1	6.7	7.2
Original value, undried	4.9	5.75	7.2	8.0	8.3
	0	1.9 Na	3.6 Na	7.8 Na	11.7 Na
Soil 7:					
5 minutes or less	3.95	4.4	5.1	5.9	7.2
2 days	4.2		5.2	6.2	7.25
5 days	4.55		5.55	6.35	7.4
13 days	4.55		5.3	6.0	7.1
21 days	4.55		5.55	6.45	7.5
35 days	4.5		5.5	6.5	7.4
Original value, undried	4.95	6.0	6.65	7.95	

Where the ultimate pH values are below 7 the difference due to drying is still probably significant even after 170 days of continuous soaking. If the drying had been continued for a longer period it is likely that the reversibility would have been somewhat less complete.

TABLE 5.—*Influence of long-continued soaking on the pH of several soils and subsoils which were oven-dried after an addition of base, compared to the same soils soaked continuously without drying and also the same kept alternately wet and air-dry*

Soil	M. E. of base	Treatment	Original pH of moist paste after 2 days with base	pH of samples after soaking—			
				30 minutes	43 days	127 days	171 days
Holston sandy loam.	5.4 Ca	Oven-dry	7.9	6.8	7.3	7.8	7.6
	5.4 Ca	Continuously wet	7.9		7.4	7.6	7.5
	5.4 Ca	Alternately wet and air-dry.	7.9		7.2	7.5	7.6
Purdy silt loam	5.4 Ca	Oven-dry	6.4	5.35	6.35	7.3	7.0
	5.4 Ca	Wet	6.4		6.9	7.05	7.0
	5.4 Ca	Wet and air-dry	6.4		6.9	6.7	6.6
Dekalb clay subsoil	5.4 Ca	Oven-dry	5.3	4.4	4.6	4.9	5.0
	5.4 Ca	Wet	5.3		5.0	5.25	5.25
	5.4 Ca	Wet and air-dry	5.3		5.0	5.1	5.2
Cavode silty clay subsoil.	5.4 Ca	Oven-dry	6.5	5.0	5.5	6.2	6.0
	5.4 Ca	Wet	6.5		6.2	6.4	6.4
	5.4 Ca	Wet and air-dry	6.5		6.1	6.2	6.1
Gilpin clay loam subsoil.	5.4 Ca	Oven-dry	7.8	6.8	6.6	6.9	7.0
	5.4 Ca	Wet	7.8		7.05	7.05	7.05
	5.4 Ca	Wet and air-dry	7.8		7.15	7.35	7.5
Dekalb clay loam subsoil.	4 Na	Oven-dry	5.7	4.8	5.2	5.45	5.7
	4 Na	Wet	5.7		5.5	5.7	5.8
	4 Na	Wet and air-dry	5.7		5.3	5.45	5.7

ALTERNATE SOAKING AND DRYING

Table 6 shows the results of alternate soaking and drying on the pH values of several soils without added base, and for one soil with several increments of Ba added. All differences shown are small and probably insignificant, indicating that the initial drying has given essentially the minimum pH value.

TABLE 6.—*Influence of alternate soaking and drying; each drying was for approximately 5 days*

Soil No.	M. E. of bases	pH of samples dried and soaked—		
		Once	Twice	Three times
1	None	4.3	4.2	4.15
2	do	4 9	4.85	4.8
3	do	5.1	5.05	5.0
5	do	4.9	4.95	5.0
7	do	4.0	4.05	4.0
8	do	3.85	3.80	3.85
		pH of sample soaked 30 days, then dried		
	1.8 Ba	4.35	4.4	
	3.6 Ba	4.80	4.75	
7	7.2 Ba	5.9	5.65	
	12.7 Ba	7.1	7 0	
	16.3 Ba	7.8	7.65	

INFLUENCE OF DRYING ON THE EXCHANGE CAPACITY AND RECOVERY OF ADDED BASES

Table 7 shows the exchange relations obtained on two soils with bases added and dried as described in previous sections. In the case of the surface soil it appears that a small amount of some bases, both Ba and Na, may have become fixed by the drying although most have been readily recovered by overnight leaching with the normal ammonium acetate. Recovery of bases seems essentially complete in the case of the clay subsoil. The base exchange capacity as measured by retention of the ammonium ion may be slightly decreased with

TABLE 7.—*Influence of oven drying upon the apparent exchange capacity and the recovery of added bases by neutral NH_4 acetate leaching*

SOIL 3—SILT LOAM SURFACE SOIL

Base added, M. E. per 100 gm.	Treatment after addition of base	Soil pH from buffer curve	Milliequivalents		Exchange bases, M. E.	Recovery of added bases		Bases not recovered, M. E.
			Exchange capacity	Apparent exchange H+		M. E.	Percent	
0	Air-dry	5.05	10.8	4.3	6.5			
0	Oven-dry	4.4	10.6	4.5	6.1			
2.5 Ba	do	5.05	9.5	1.8	7.7	1.6	64	0.9
3.8 Ba	do	5.5	10.3	1.0	9.3	3.2	84	.6
7.6 Ba	do	6.65	10.4		12.0	5.9	78	1.6
2.0 Na	do	5.2	10.0	2.6	7.4	1.3	65	.7
3.9 Na	do	5.75	10.0	.7	9.3	3.2	82	.7
7.8 Na	do	6.7	10.0		12.6	6.5	83	1.3

SOIL 7—CLAY SUBSOIL

Base added, M. E. per 100 gm.	Treatment after addition of base	Soil pH from buffer curve	Milliequivalents		Exchange bases, M. E.	Recovery of added bases		Bases not recovered, M. E.
			Exchange capacity	Apparent exchange H+		M. E.	Percent	
0	Air-dry	5.0	20.5					
0	Oven-dry	4.0			15.6			
3.1 Ba	do	4.7	19.8	1.4	18.4	2.8	90	.3
4.7 Ba	do	5.1	21.6		21.1	5.5	115	
9.4 Ba	do	6.3	19.2		22.7	7.1	76	2.2
2.5 Na	do	4.7	19.5	1.2	18.3	2.7	108	
3.8 Na	do	5.0	20.2	.6	19.6	4.0	105	
7.6 Na	do	5.8	19.6		22.4	6.8	89	.84

both soils (excess ammonium acetate was removed by leaching with ethyl alcohol).

Much more detail about the relative degree of fixation of various ions by drying may be found in other work [3] (*23*). The data here are sufficient to show that drying may introduce certain complications into the measurements of exchange capacity and exchangeable bases.

INFLUENCE OF DRYING ON THE RELEASE OF H+ TO A NEUTRAL SALT

Successive additions of neutral normal $BaCl_2$ solution were shaken with two different soils to determine the release of H+. After shaking and centrifuging, the solution was poured off and back titrated to determine the release of H+. When an essential end
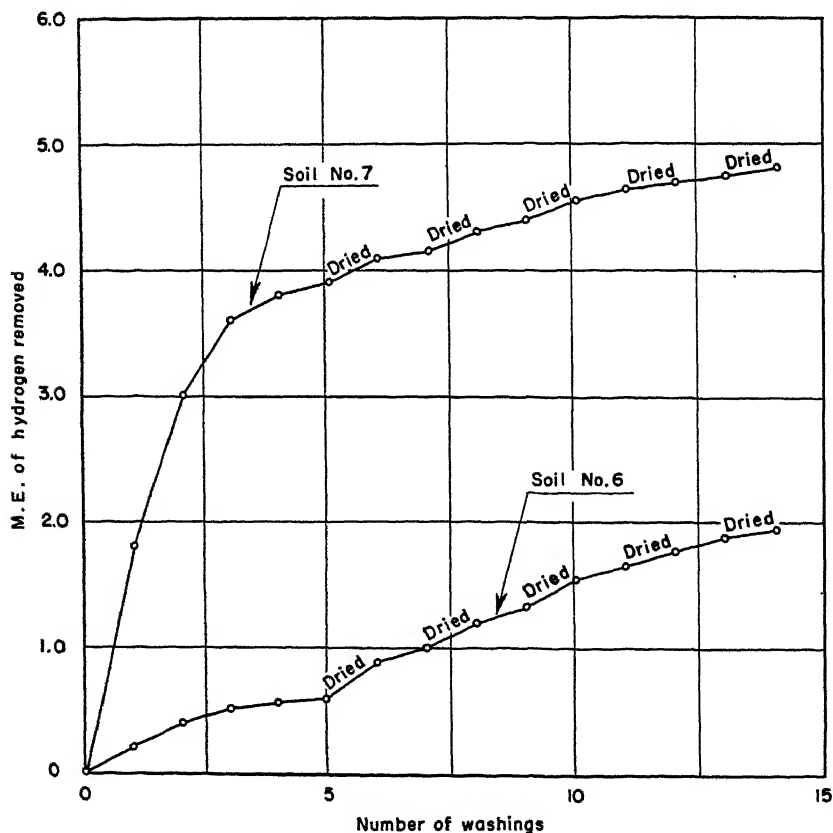
FIGURE 3.—Influence of drying on the release of H+ to a neutral salt solution of $BaCl_2$. Soil 7, a slack water clay subsoil, shows a distinct but small percentage increase in H+ release. Soil 6, a loam subsoil, shows a somewhat greater actual and a much greater percentage increase in H+ release.

[3] PAGE, J. B. THE RELATION OF IONIC SIZE TO THE FIXATION OF POTASSIUM AND OTHER CATIONS BY COLLOIDAL CLAY. Ohio State Univ. Doctoral Diss. 32. 1940.

point was approached after five washings, the soil and salt were oven-dried, redispersed, and the H+ titrated. As shown in figure 3, the successive dryings which followed caused an additional release of H+. The total and the percentage increase were much greater for the soil with lowest exchange capacity and less clay.

INFLUENCE OF DRYING ON SOIL BUFFERING AGAINST DIFFERENT CATIONS

It is evident from the tables that drying eliminates part of the differences in buffering commonly associated with the different cations. With Na the decrease in pH due to drying is consistently greater than with Ba. This would, of course, tend to bring Na and Ba buffer curves closer together because, as is well known, Na normally increases the pH of a soil more than an equivalent amount of Ba.

The drying influence on cation differences is shown more clearly for two soils in figures 4 and 5. Here the drying has been continued long enough to approach the minimum pH, which is not true of all the values shown in the tables.
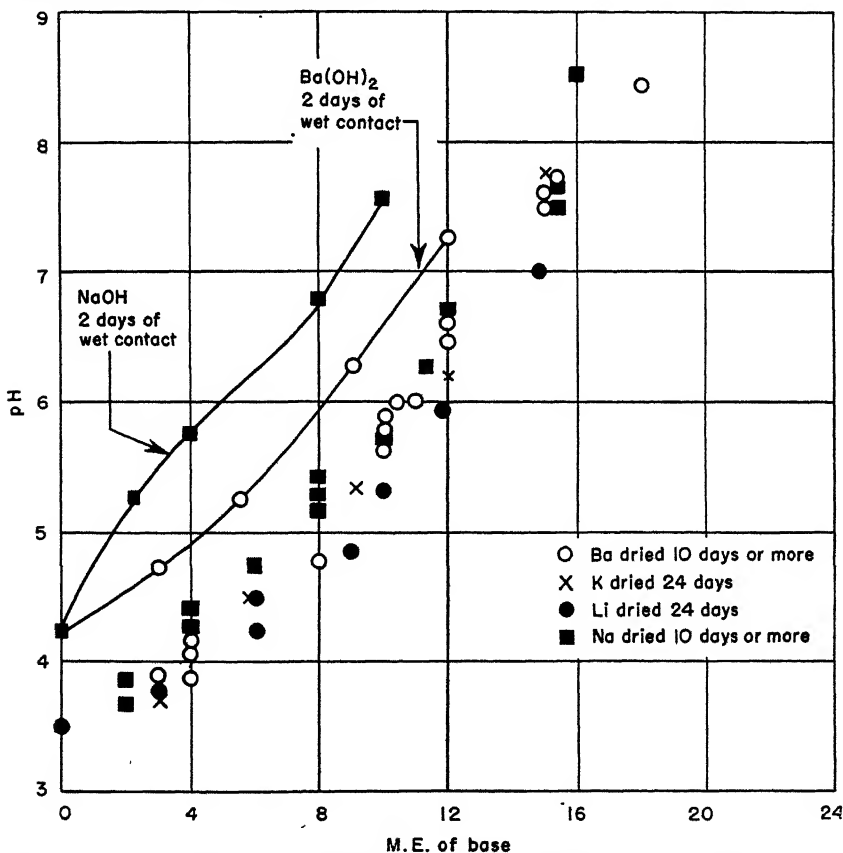
FIGURE 4.—Buffering of a Dekalb loam subsoil against several bases. Note the distinctly different curves for Ba and Na in moist suspensions but the overlapping of values after drying. It seems evident that drying eliminates much of the difference between bases.
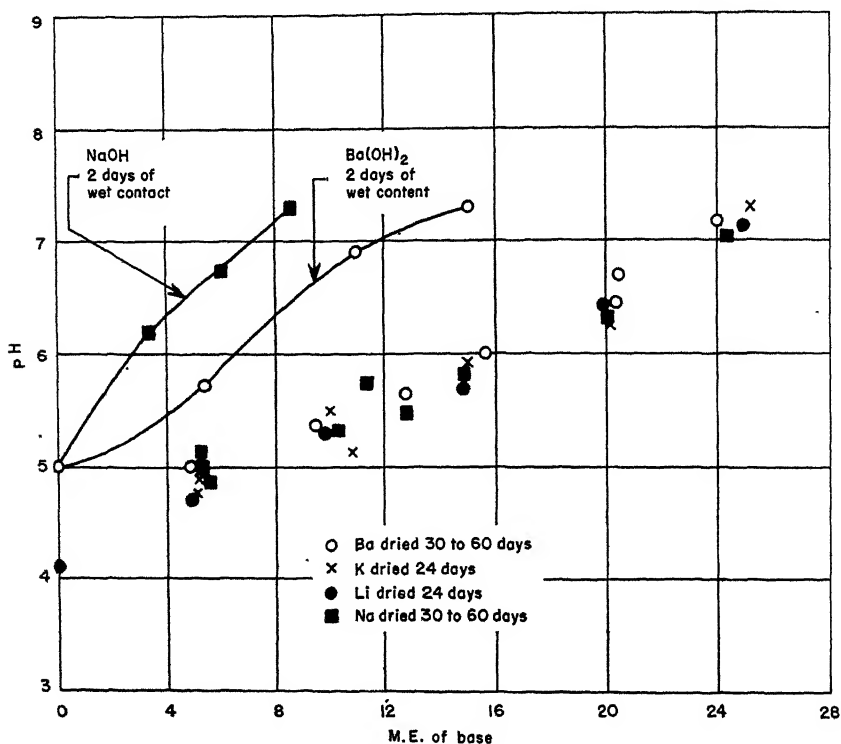
FIGURE 5.—Buffering of Purdy silt loam surface soil against several bases. Note the distinctly different curves for Ba and Na in moist suspensions but the overlapping of values after drying. Consistent differences between the bases do not seem to be evident after drying.

In figure 4 the soil buffering is dominated by the clay; in figure 5 by organic matter. With both soils the differences among Na, Li, K, and Ba are small and rather inconsistent. The writers suspect that the variations shown are mainly inherent errors, because 0.2 and 0.3 pH variations are common when an attempt is made to duplicate a particular dry buffer curve at a different date. Some of the sources of this error are shown to be associated with a lack of complete or equilibrium drying, some with the degree or time of grinding or shaking. In any case, it is clear that drying eliminates most of the differences among bases as they affect the pH of soil. This reduces soil buffering to a more or less absolute property which may be defined independently of the base used.

SOME FACTORS WHICH MAY CAUSE VARIATIONS IN THE DRYING EFFECT

DRYING TIME

Many of the data reported in the early part of this paper are based on a more or less indefinite time of drying. This appeared justified by the belief that a few days at 105° C. would invariably result in

constant weight and little likelihood of other changes. This assumption gave results that were satisfactory in showing some of the general influences of drying, but many unexpected and variable detailed results led to a more critical examination of the effect of time.

Table 8 shows that a few days of drying are not enough to give minimum pH values. There is a trend downward which extends over a period of 20, 30, or more days depending on the soil. When much organic matter is involved the time required to attain a minimum value seems to be longer than when the buffering material is clay.

TABLE 8.—*Influence of time of drying on pH of several different soils treated with bases; the bases were added in solution and drying was started after about 2 days of contact*

Soil	M. E. and kind of base	pH of soil after drying for number of days indicated												
		1	2	3	4	7	10	14	15	18	27	32	48	64
Ashby silt loam subsurface	11.0 Na	6.6	----	6.45	6.2	5.8	6.0	5.8	----	----	----	----	----	----
	15.8 Na	7.6	----	7.6	7.6	6.85	6.8	6.55	----	----	----	----	----	----
	14.0 Ba	7.0	----	6.85	6 45	6.05	----	----	----	----	----	----	----	----
Calvin silt loam surface	4.9 Na	7.0	----	6.8	6.35	5.5	5.55	5.5	----	----	----	----	----	----
	9.7 Na	7.9	----	8.1	7.2	6.7	6.5	6.5	----	----	----	----	----	----
	3.5 Ba	6.2	----	5.8	----	5.4	----	----	----	----	----	----	----	----
	7.0 Ba	7.05	----	6.9	----	6.5	----	----	----	----	----	----	----	----
	10.5 Ba	7.9	----	7.8	----	7.5	----	----	----	----	----	----	----	----
Dekalb sandy clay loam subsoil	2.0 Na	----	4.45	----	----	----	4.0	----	----	3.87	3.95	----	----	----
	6.0 Na	----	5.45	----	----	----	5.0	----	----	4.9	4.70	----	----	----
	8.0 Na	----	5.95	----	----	----	5.75	----	----	5.5	5.3	----	----	----
	2.9 Li	----	----	----	----	----	----	----	3.8	----	----	3.7	3.9	3.7
	5.9 Li	----	----	----	----	----	----	----	4.2	----	----	4.1	4.1	4.15
	11.9 Li	----	----	----	----	----	----	----	6.0	----	----	5.9	6.1	5.85
	12.1 K	----	----	----	----	----	----	----	6.7	----	----	6.4	6.6	6.05
Blago silt loam high organic	20 Na	----	5.45	----	4.9	----	----	----	----	----	----	----	----	----
	50 Na	----	6.95	----	6.4	----	----	----	----	----	----	----	----	----
	70 Ba	----	7.05	----	6.7	----	----	----	----	----	----	----	----	----
Purdy silty clay	4.9 Li	----	----	----	----	----	----	----	5.1	----	----	4.8	5.0	4.7
	24.7 Li	----	----	----	----	----	----	----	7.7	----	----	7.2	7.45	7.1
	5.0 K	----	----	----	----	----	----	----	5.1	----	----	4.95	5.0	4.8
	15.0 K	----	----	----	----	----	----	----	6.6	----	----	6.1	6.6	5.9

The complete explanation of this long time requirement is somewhat uncertain, but it may be related to the loss of the last remnants of water from the sample. Table 9 shows that a significant loss of weight by the type of samples being studied extends over a considerable period of time. This water would, of course, be concentrated on colloidal surfaces as would the various cations and might thus exert an influence out of all proportion to the quantity of water involved.

TABLE 9.—*Influence of time of drying on loss of weight by 20-gram samples of soil treated with bases*

Soil	M. E. of base	Cumulative percent loss of weight by oven drying for number of days indicated					
		2	4	8	16	24	36
Dekalb loam subsoil	15 Na	Start	0.028	0.061	0.127	0.125	0.127
	12 K	---do---	.038	.102	.149	.150	.149
Purdy silt loam surface	20 Na	---do---	.183	.400	.700	.811	.902
	17 K	---do---	.177	.403	.767	1.090	1.196

GRINDING

It has not been considered desirable to grind samples for the pH determinations, but tests were made to find out whether this might be a source of error. Table 10 shows that grinding to pass a 40-mesh screen can cause a definite change of pH. The effect may be either an increase or a decrease depending on the nature of the soil (*2*). Grinding should, therefore, be avoided.

TABLE 10.—*Changes in pH of oven-dried samples caused by grinding*

Soil	Base M. E	pH of slaked samples	pH of ground samples	Change due to grinding
Dekalb sandy clay loam subsoil	12 Na	7. 85	8. 1	+0. 25
	10 Na	6. 6	6. 9	+. 30
	8 Na	5. 95	5. 95	None
	6 Na	5 45	5. 15	—. 30
	4 Na	4 65	4. 9	+. 25
	2 Na	4 45	4. 6	+. 15
	14 Ba	7. 95	7. 75	—. 20
	12 Ba	7. 3	7. 15	—. 15
	10 Ba	6. 3	6. 1	—. 20
	8 Ba	5. 2	5 25	+. 05
	6 Ba	4. 75	4. 8	+ 05
	4 Ba	4. 45	4. 45	None
	3 Ba	4. 2	4. 3	+. 10
Blago silt loam	50 Na	7. 5	6. 95	—. 55
	40 Na	7. 1	6. 5	—. 60
	30 Na	6. 35	5. 8	—. 55
	20 Na	5. 65	5 45	—. 20
	80 Ba	7. 6	7. 3	—. 30
	70 Ba	7. 3	7. 05	—. 25
	60 Ba	6. 85	6. 05	—. 80
	40 Ba	5. 8	5. 7	— 10
	30 Ba	5. 35	4. 75	—. 60
	20 Ba	4. 85	4. 25	—. 60
	10 Ba	4. 0		

TIME OF CRUSHING

Although thorough grinding is unnecessary, a sample dried from a suspension must be crushed in order to give a good mixture and to be suitable for the pH test. It did not seem that the exact procedure for crushing should make much difference, but comparisons shown in table 11 indicate that the time of crushing can influence the results.

TABLE 11.—*Influence of time of crushing on the pH of Dekalb sandy clay loam subsoil dried with different increments of base*

Base M. E.	pH of soil paste			
	Crushed after 1 day, then dried for—		Crushed only after drying for—	
	30 days	44 days	31 days	45 days
3.0 Na	4. 5	4. 5	4. 6	4. 8
7.1 Na	5. 3	5. 2	5. 3	5. 3
11.1 Na	6. 45	6. 3	6. 8	6 55
15.2 Na	8 3	8 1	8. 4	8. 3
2.95 K	4. 6	4. 25	4. 4	4. 55
7.1 K	4. 9	4. 8	5. 2	4. 95
10.9 K	5. 9	5. 8	6. 1	6 0
15.1 K	7. 9	7. 8	8. 4	8. 4

For some reason, crushing after 1 day of drying resulted in lower values than when the soil mass remained intact until the end of an extended drying period.

<div align="center">TEMPERATURE OF DRYING</div>

Since drying at a fixed temperature such as 105° C. is somewhat arbitrary, a higher temperature was used with some samples to determine whether this might cause an even lower pH. But the differences in pH values at 150° and 105° were rather small and inconsistent. In some cases a lower value was obtained, in others a higher one. A few comparisons are summarized in table 12. It appears that with sufficient time a true minimum is reached at about 105°, and the 150° temperature cannot be expected to cause any additional lowering of the pH. A small increase in pH with some samples appears significant and may be due to the volatilization of certain acid constituents at the 150° temperature.

TABLE 12.—*A comparison of the pH values obtained after drying at 105° C. and at 150° C.*

Soil	Amount and kind of base added, M. E.	pH after drying at—			
		105° C.		150° C.	
		15 days	Approximately 5 days	28 days	Approximately 5 days
Dekalb sandy clay loam	⎧3 K	3.8		4.5	
	⎪6 K	4.5		4.8	
	⎪9 K	5.4		5.4	
	⎨12 K	6.6		6.5	
	⎪15 K	7.9		8.0	
	⎪3 Na	3 8		4.4	
	⎩11.2 Na	6.0		6.2	
Litz silt loam surface	None		5.2		5.3
Do	do		5.5		5.5
Frederick subsurface	do		6.0		5.9
Dekalb sand	do		4.0		4.2
Calvin subsurface	do		4.0		4.1
Calvin subsoil	do		3.6		3.7
Ashby silt loam surface	do		4.1		4.1
Ashby silt loam subsurface	do		4 0		4.1
Do	do		4.0		4.1
Corydon clay subsoil	do		6.9		6.0
Ungers silt loam surface	do		4 4		4.7
Ungers silt loam subsoil	do		4.5		4.5

<div align="center">GENERAL DISCUSSION</div>

Results presented show that with the varied soils and soil materials studied drying causes a considerable change in soil buffering relationships, as well as in the observable aggregation. It appears that the buffer curve of any soil may be thought of as being defined within two extremes. The weakest buffering is that obtained by placing a soil and a base in contact in a dilute suspension. After approximately 2 days of contact a fairly well defined curve is obtained which is ordinarily accepted as the buffer curve for the soil with the particular base used. This is also ordinarily the most dispersed condition for the soil. The curve is invariably higher on the pH scale for bases

like lithium and sodium hydroxides than for calcium or barium. This also corresponds with the degree of dispersion.

If, after essential equilibrium has been attained, the soil-base suspension is thoroughly dried, then redispersed in water, a different buffer curve is obtained. The soil is also more completely aggregated. This curve invariably falls completely below the ordinary curve on the pH scale and shows stronger buffering. The least actual pH change caused by the drying is usually for the soil with no base added. Here, too, there is likely to be the least difference in aggregation.

Soaking in water following drying causes increases in the pH values. It also causes the soil to tend to redisperse. The soil with no extra base added usually returns to essentially the original value within 30 days, but with base additions the pH may not entirely return even after 6 months of continuous soaking; that is, the most quickly reversible process seems to be that which involves the soil without freshly added base. Drying apparently changes some relation between the soil and fresh base in such a way that it is only very slowly and often incompletely reversible. This change would seem to consist of a closer association of the basic ion with the exchange complex much the same as aggregation is favored by the drawing together of the colloidal surfaces. A closer attachment would naturally be expected as a result of drying, and it also seems reasonable that a hysteretic effect might result from drying, as in many physical and chemical phenomena. This might be thought of as a simple mechanical entrapment. The extreme smallness of the H+ ion when dehydrated would explain why it might completely escape entrapment and be free to contribute to a somewhat increased concentration of hydrogen ion, or lower pH. In the case of base-free soil (acid leached or electrodialized) the effect of drying on pH might be accounted for by a drawing together of colloidal surfaces so closely that some negative charges would become ineffective. Rehydration by soaking would reverse this process, as shown by the increase of pH values.

When soils are limed in the field the process of aging, primarily wetting and drying, would bring bases into a closer association with colloidal surfaces just as indicated in the laboratory. This would help to account for the fact that a liming factor has been necessary to convert laboratory buffer values into field liming recommendations. The dry buffer curve as described gives an indicated buffering which is of the proper order of magnitude to account for a liming factor of 1.5 to 2.0. It seems likely that many of the recognized variations of soil pH in the field both before and after liming are within the zone defined by the moist suspension and the dry buffer curve. The momentary pH would be expected to reflect the relationship of the soil and base with respect to the cycle of hydration or dehydration. The lowest pH would be the point of closest attachment between soil and base, and also between colloidal surfaces. It would, therefore, seem to correspond to the highest aggregation.

Since drying completely alters the shape of a soil buffer curve, giving essentially a straight line relationship between pH and milliequivalents of base added in many cases, there seems to be some doubt as to whether the shape of the moist buffer curve is very significant in determining such properties as exchange capacity. The shape changes

again on hydration but does not seem to have a well-defined form which can be readily interpreted.

Drying was also shown to influence the amount of exchangeable $H+$ as determined with a neutral salt ($BaCl_2$). With neutral ammonium acetate leaching it was shown that for two soils the retention of NH_4 ions as a measure of exchange capacity was little altered by drying. The bases recovered were sometimes in excess of the NH_4+ retention, although the pH of the soils with bases was well below 7. Drying thus caused an apparent contradiction by this method of determination. It seems likely that other methods of determining exchangeable $H+$, exchangeable bases, and exchange capacity might be influenced in a manner similar to that shown for the buffer curve, the $BaCl_2$, and the NH_4Ac methods. A consideration of the hydration factor and its influence on the tightness with which bases are held might help to reconcile some of the discrepancies among methods and explanations noted in the literature. An apparent build-up of exchange, for instance, could be accounted for by a closer association between the cation and the colloid, at least by certain methods of determination.

The fact that the drying effect upon soil pH and buffering is similar for a variety of soils and subsoils, for one electrodialyzed clay with high exchange capacity, for pure kaolinite and for bentonite seems to indicate that the phenomenon is of very general application. This is further emphasized by the comparisons among bases which show that Na, Ba, K, Li, and Ca all respond similarly. These similarities among soils and among the bases suggest that the drying influence can be classed as a typical hysteresis loop in buffer curves, and is probably traceable to some sort of closer association between cation and colloidal charges.

There are definite indications from observations and from actual measurements that drying increases soil aggregate stability (*16*) as well as acidity. This aggregation may be related to the indicated drawing together of clay surfaces and increases in acidity. At least, this should be investigated further along with the detailed physical-chemical relationships mentioned. This suggests that the aggregation and the increased acidity may be related through a common cause. The cause which appears most likely is the drawing of colloidal surfaces so close together by drying that molecular forces prevail and tend to prevent a complete rehydration. This paper does not offer an answer to many of the detailed, quantitative questions which are raised in considering the effect of drying on soil acidity and aggregation. Much more experimental work and theoretical study appear needed. The interrelation of drying, hydration, base exchange, acidity, and soil aggregation seem to appeal almost equally to the theoretical and the practical soil scientist.

SUMMARY

A review of the literature suggests that many questions regarding soil buffering relationships are not yet answered. The effect of drying is introduced as one approach to answering some of the theoretical and practical questions involved.

pH determinations of many varied samples show that oven drying

definitely increases the acidity as compared to that of moist or air-dried materials. The effect is most pronounced when freshly added bases are involved but is also evident in soils leached free of bases. An increased buffering results from the greater effect with added bases. Alternate soaking and drying has little more effect than a single drying. The reversibility of the effect of drying with fresh bases is very slow and probably never complete, at least with some soils. When no new bases are added the pH usually returns to the original value in 30 days or less.

Oven drying had only a small influence on the exchange capacity or exchangeable bases in one surface silt loam and one clay subsoil by ammonium acetate leaching; but it resulted in the peculiar situation of having a higher content of bases than the exchange capacity held by the soil at pH values well below neutrality. Oven drying of soils also increased the release of H+ to a neutral salt.

Buffering comparisons involving Li, Na, K, Ba, and Ca show that drying eliminates most if not all of the differences normally shown by these different cations.

A consideration of factors causing variations in the effect of drying shows that drying of 20, 30, or even more days is necessary to give a minimum, reproduceable pH. Grinding after drying increased the pH in all cases with a subsoil clay and decreased it with a high organic surface soil. Crushing in the early stage of drying caused the pH to go somewhat lower than when crushing was delayed until after a prolonged drying. A temperature of 150° C. gave results only slightly different from those at 105°, and in most cases the change was a small increase in pH.

A general discussion points out that the drying effect may help to reconcile some apparent contradictions of methods and results in the literature. It also appears to have possible application both in practical and in theoretical studies. The similarity of results with the various cations offers some opportunities for establishing an absolute buffering value independently of the cation used. In field liming the indicated dry buffering may help to account for the necessity of a "liming factor" to convert laboratory values to field requirements.

The fact that aggregation and acidity are both increased by drying suggests that the drawing together of colloidal surfaces may be the fundamental cause of both phenomena. Much more study is needed to clarify this point and other details of the interrelations among drying and rehydration, buffering, and aggregation.

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HATCHABILITY IN RHODE ISLAND REDS AS AFFECTED BY AGE OF PARENTS [1]

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INTRODUCTION

Hatchability in individual females or in pens is usually measured by the percentage of fertile eggs that hatch. This character is vitally important in the economical propagation of the flock. Females that give low hatchability have very little value as reproducers and are usually discarded even though they may possess many desirable characters as far as egg production is concerned. There is rather general agreement among investigators that hatchability is an individual trait and that both heredity and environment have important effects.

Landauer (*10*) [2] in 1941 reviewed the problem thoroughly so that any extensive review of the literature is unnecessary. Insko, Steele, and Wightman (*9*) have brought the literature up to date (1947) and have presented new data pointing to a decline in hatchability as females grow older.

EXPERIMENTAL RESULTS

The hatching records of pedigreed Rhode Island Reds bred at the Massachusetts Agricultural Experiment Station for high fecundity have been examined for the 15-year period from 1933 to 1947. Only individuals laying 10 or more fertile eggs have been included. It is the opinion of the writers that a considerable volume of data secured over a period of time presents a truer picture of the actual than would be the case with *fewer data subjected* to detailed statistical treatment.

AGE OF MALE IN RELATION TO HATCHABILITY

Male breeders ranged in age from about 10 months to 48 months. Males were grouped into four classes: cockerels (up to 12 months); yearlings (up to 24 months); 2-year-olds (up to 36 months); and 3-year-olds (48 months +). Most of these males were mated to females of various age ranges; thus the relation of age of male to hatchability may be examined.

The data show that the mean hatchability of the four classes was as follows: 135 cockerels, 83.64 percent; 143 yearlings, 80.67 percent; 60 2-year-olds, 82.26 percent; and 12 3-year-olds, 80.67 percent.

These gross data suggest that the age of male breeders has very little, if any, effect on hatchability

A more accurate measure of the effect of age of males lies in the comparison of the records of identical males in successive years not shown in tables. These males were mated to similar age groups of females. Eight males which in the breeding pens had a mean hatchability of 84.6 percent as cockerels had a mean hatchability of 81.2 percent as yearlings. Five males were used in 3 successive years with the following hatchability records: As cockerels, 86.3 percent; as yearlings, 73.5 percent; and as two-year-olds, 90.2 percent. Seventeen males placed in the breeding pens first as yearlings gave a mean hatchability of 83.3 percent this first year, 79.0 percent the next year. Four males were tested first as yearlings and for the 2 following years. Their hatchability records were 89.8, 84.3, and 82.6 percent.

These rather limited data suggest that hatchability declines at a very slow rate as males grow older. This fact is in accord with previous observations (*5*, *11*, *4*, *6*) that hatchability is governed largely by inheritance which should not change with age, but that certain unknown physiological factors may also be in operation.

The complete data for the 15-year period are presented in table 1.

TABLE 1.—*Hatchability and age of breeders for the 15-year period*

Mating	Sires	Dams	Average hatchability
	Number	*Number*	*Percent*
Pullet ✕:			
Cockerel	87	518	83.8
Yearling	43	234	81.9
2-year-old	16	66	82.7
3-year-old or older	2	3	90.1
Totals and means	148	821	83.2±0.8607
Yearling ✕:			
Cockerel	26	97	84.8
Yearling	69	342	81.5
2-year-old	25	114	84.9
3-year-old or older	5	17	83.3
Totals and means	125	570	82.9±0.8842
2-year-old ✕:			
Cockerel	17	42	83.4
Yearling	20	48	78.5
2-year-old	12	36	80.9
3-year old or older	5	17	74.2
Totals and means	54	143	80.2±1.9542
3-year-old or older ✕:			
Cockerel	5	8	75.5
Yearling	11	15	74.5
2-year-old	7	10	74.1
3-year-old or older			
Totals and means	23	33	74.6±2.4902

AGE OF FEMALES IN RELATION TO HATCHABILITY

The means for the four age groups of females are given together with their standard error. It will be observed that a significant drop in hatchability first appeared in females that were 3 years old or older. Since there were only 33 females in this oldest group the decline in the mean does not afford conclusive evidence.

THE CHANGE WITH AGE IN THE HATCHABILITY OF IDENTICAL FEMALES

A total of 131 females gave a mean hatchability of 87.6 percent in their pullet year and 81.8 percent in their yearling year. Of 25 females used in 3 successive years, the mean hatchability was 91.8 as pullets, 84.3 as yearlings, and 83.0 percent as 2-year-olds. Seventy females were first placed in the mating pens as yearlings and were again used for breeding when 2-year-olds. Their hatchability as yearlings was 87.1 percent and as 2-year-olds, 77.9 percent. Twenty females that began their test as yearlings were tested over 3 years with a mean hatchability of 93.0 percent, 87.0 percent, and 74.4 percent.

In general, there was more decline in hatchability as age increased when identical females were examined than was shown in table 1 for the whole female population. This difference would be expected because a considerable number of pullet breeders gave low hatching records which eliminated them from further testing and reduced the mean for the complete pullet group. This same situation also applies to females that began their test as yearlings. In general, these data indicate that pullet or yearling breeders are likely to show a decline in hatchability with age.

IDENTICAL MATINGS IN SUCCESSIVE YEARS

Few identical matings are available for study of the effect of age of both parents on hatchability. Five identical matings were used in which the same parents were placed in the mating pens as cockerels and pullets and again as yearlings. The mean hatchability in these cases was 81.8 percent for the first year and 76.1 percent for the second year.

Five matings in which the sires were 2-year-olds and the dams were yearlings in the first year and in the second year both parents were 1 year older gave means of 93.6 percent and 80.1 percent hatchability respectively. These limited data from identical matings indicate something of a tendency for hatchability to decline as parents grow older.

EMBRYONIC MORTALITY IN RELATION TO AGE OF PARENTS

Dead embryos were grouped into four general classes with respect to age at death. Early deaths include those from the first to about the fifth day of development; late deaths include those from about the sixth to the eighteenth day; and very late deaths those that occurred after the eighteenth day up to full term. Very late live embryos include those developed to full term, remaining alive on the twenty-second day but failing to emerge from the shell.

Early embryonic mortality was practically the same from pullet and yearling mothers, about 4.3 percent (table 2). This means that slightly more than 4 percent of the fertile eggs gave rise to embryos that died early in incubation. There was some increase in mortality of embryos from 2-year-old mothers and a very marked increase in those from the very old hens. Table 2 shows also that this tendency for embryos to die early generally increased when the older males were mated to 2-year-old or older hens. Any effect of age of male does not appear when pullet or yearling mothers were used.

TABLE 2.—*Percentage of embryonic mortality for the 15-year period*

Mating	Sires	Dams	Average embryonic mortality		
			Early	Late	Very late [1]
	Number	*Number*	*Percent*	*Percent*	*Percent*
Pullet ✕:					
Cockerel	87	518	4.1	9.4	2.3
Yearling	43	234	4.7	10.4	2.2
2-year-old	16	66	5.0	10.6	1.3
3-year-old or older	2	3	1.1	4.7	2.0
Totals and means	148	821	4.3±0.2735	9.7±0.6397	2.2±0.1903
Yearling ✕:					
Cockerel	26	97	2.8	8.1	2.9
Yearling	69	342	5.1	9.8	3.1
2-year-old	25	114	4.0	7.7	1.8
3-year-old or older	5	17	4.6	8.4	2.8
Totals and means	125	570	4.4±0.3396	9.0±0.5325	2.8±0.2896
2-year-old ✕:					
Cockerel	17	42	4.5	7.7	3.7
Yearling	20	48	5.0	13.5	2.7
2-year-old	12	36	9.7	7.3	1.0
3-year-old or older	5	17	9.1	12.2	2.9
Totals and means	54	143	6.3±0.6690	10.2±1.1762	2.7±0.5003
3-year-old or older ✕:					
Cockerel	5	8	7.1	12.2	3.2
Yearling	11	15	9.0	12.8	2.1
2-year-old	7	10	12.9	8.5	3.9
3-year-old or older	0	0	------	------	------
Totals and means	23	33	9.8±1.7205	11.4±1.8938	2.9±0.6945

[1] Fully developed embryos that may or may not break the shell.

There was no evidence to indicate any significant effect of age of either parent on late or on very late embryonic death rate.

FULL-TERM LIVE EMBRYOS THAT FAIL TO EMERGE

Specific causes for the failure of live embryos to emerge from the shell are but little understood. A small number of lethals that produce morphological effects have been described, and the influence of position has been extensively studied by Byerly and Olsen (*2*), Hutt (*7*), Insko, and Martin (*8*), and many others. The work of Byerly and Olsen (*1*), Dove (*3*), and Waters (*12*) raises doubt concerning the importance of malpositions as factors in hatchability.

Table 3 gives the complete data on the percentage of full-term embryos that failed to emerge even though the embryos were alive on the twenty-second day. The relative incidence ranges from 1 to 9 percent, but the average is rather low. There is considerable evidence to suggest that when the parents are older, the incidence is higher. This fact accounts in part for the decline in hatchability with increase in age of parents.

The general results of the study of embryonic mortality over 15 years seem to point to a higher early embryonic death rate from matings of older parents, to no significant relation of age of parents to late or very late embryonic death rate, and to an increase in the percentage of live embryos that fail to emerge in eggs from older parents. These facts largely explain the observed decline in hatchability as birds grow older, but it seems probable that unrecognized physiological factors also are operating.

TABLE 3.—*Percentage of live full-term embryos that failed to emerge during the 15-year period*

Mating	Sires	Dams	Average
	Number	*Number*	
Pullet ✕:			
Cockerel	30	202	1.4
Yearling	17	124	2.0
2-year-old	7	30	1.0
3-year-old or older	2	3	2.2
Totals and means	56	359	1.5±0.2012
Yearlings ✕:			
Cockerel	8	27	4.6
Yearling	22	125	1.9
2-year-old	12	47	3.3
3-year-old or older	2	4	2.4
Totals and means	44	203	2.8±0.3954
2-year-old ✕:			
Cockerel	6	13	2.0
Yearling	3	7	2.1
2-year-old	7	24	1.9
3-year-old or older	2	7	4.0
Totals and means	18	51	2.2±0.5911
3-year-old or older ✕:			
Cockerel	3	5	3.4
Yearling	2	3	9.0
2-year-old	3	4	1.7
3-year-old or older			
Totals and means	8	12	4.2±1.6503

SUMMARY

A study was made of the complete hatching records of Rhode Island Red chickens bred for high fecundity over a 15-year period to discover possible relations between age of parents and hatchability. The data included 135 cockerels, 143 yearlings, 60 2-year-olds and 12 3-year-olds as male parents, and 821 pullets, 570 yearlings, 143 2-year-olds, and 33 older hens as female parents.

The following deductions were made from these data:

(1) Hatchability declines at a slow rate as males grow older and at a somewhat higher rate as females grow older.

(2) Males used in successive years exhibit some decline in hatchability.

(3) Females tested in successive years tend to show a declining hatchability.

(4) Identical matings in successive years demonstrate this effect of age.

(5) Embryonic death records show a greater incidence of early embryonic deaths from eggs of older parents as well as a higher incidence of full-term live embryos that failed to emerge.

(6) Late and very late embryonic deaths were no higher in eggs from older than from younger parents.

(7) No specific causes for the changes in embryonic losses with increased age of parents have been found.

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EFFECT OF SODIUM SULFAMERAZINE ON SALMONELLA PULLORUM [1]

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INTRODUCTION

The therapeutic value of some of the sulfonamides in reducing mortality among chicks infected with *Salmonella pullorum* has been shown by the work of Severens, Roberts, and Card,[3] by Roberts, Card, and Alberts,[4] and by others. It is well known that in some cases a tolerance to a sulfonamide may be acquired by bacteria. It is therefore important to know whether or not *Salmonella pullorum* may acquire tolerance, for if it does, the effective use of sulfonamides in the control of pullorum disease may be greatly decreased. A culture of *Salmonella pullorum* was grown in increasing concentrations of sodium sulfamerazine and chicks were inoculated with the treated and untreated cultures to determine what effect, if any, was produced by the sodium sulfamerazine.

METHODS

A culture was produced from a single bacterium. This was divided into two subcultures. One served as the control and the other was grown in a medium containing sodium sulfamerazine. The basic medium used was tryptose phosphate broth with 0.2 percent dextrose. The beginning concentration was 100 mg. of sodium sulfamerazine per 100 ml. of medium. This was increased by 10-mg. increments until the final concentration of 200 mg. was reached. The length of time between the first and final concentration was 98 days. The first inoculations of chicks with the treated and control cultures were made when the culture had been grown for 36 days in a 200-mg. concentration of sodium sulfamerazine (table 1). At the second and third inoculations the times were 268 and 275 days respectively (table 2). At the last inoculation the time was 298 days (table 3). All chicks were New Hampshires and were from pullorum-free flocks. During the first 134 days transfers were made at least daily and after that about twice each week. The control or untreated culture was transferred at the same time as the treated culture, so that at any given time the number of transfers was the same for both cultures. When transfers were first made to a higher concentration an inhibiting effect on growth occurred. This soon disappeared and in the highest

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concentration of 200 mg. per 100 ml. of medium the growth was finally as great as that of the untreated culture in normal medium. This indicates an acquisition of tolerance to sodium sulfamerazine by the treated culture. Attempts to use a higher concentration always resulted in marked inhibition of growth.

When the untreated culture was put in the 200-mg. concentration of sodium sulfamerazine, inhibition of growth occurred but it was not lethal. A concentration of 1,000 mg. per 100 ml. was lethal, but the pH was above 11 and the lethal effect may have been due to alkalinity rather than to the sodium sulfamerazine.

It should be pointed out that the concentration of sodium sulfamerazine may have changed as the bacteria increased in number. As they grew, acid was produced which lowered the pH, and some precipitation of sulfamerazine resulted. All attempts to buffer the medium failed to prevent precipitation.

All cultures were incubated at 37° C. and examined after 12 and 24 hours. The degree of growth in cultures was observed by photonephelometer turbidity readings and by plate counts. For inoculation, both treated and untreated cultures were standardized to contain the same number of bacteria per dose. Sodium sulfamerazine was administered in the feed beginning when the chicks were 1 day of age.

RESULTS

The first test of untreated and treated cultures was made with day-old chicks orally inoculated with ¼ ml. of 24-hour cultures of *Salmonella pullorum*. All the chicks were fed the same ration containing 0.5 percent sodium sulfamerazine. The treated culture had been grown in the final concentration of 200 mg. of sodium sulfamerazine in 100 ml. of medium 36 days before the inoculation of chicks. The percent mortality during the 21-day period was 29.4 among the chicks inoculated with the untreated culture and 13.7 for the group inoculated with the treated culture (table 1).

TABLE 1.—*Mortality of chicks orally inoculated at 1 day of age with untreated and treated [1] cultures*

Chicks (number)	Inoculated with culture—	Ration No.	Mortality in 21 days	Average weight of survivors at 21 days
			Percent	*Grams*
51	Untreated	391 +0.5 percent Ssm [2]	29.4	118
51	Treated	do	13.7	135

[1] Grown in medium with a final concentration of 200 mg. of sodium sulfamerazine in 100 ml. for 36 days
[2] Sodium sulfamerazine.

The treated culture was continued in the 200-mg. concentration medium and the second and third tests were made 232 and 239 days later. In these tests the chicks were subcutaneously inoculated at 3 days of age with ¹⁄₁₀ ml. of 24-hour cultures. In addition to the two groups of chicks that received 0.5 percent sodium sulfamerazine in the feed (ration 391), two other groups were inoculated which did not receive sodium sulfamerazine in the feed. The results are given in table 2. Mortality was significantly greater ($P<0.01$) among

TABLE 2.—*Mortality of chicks subcutaneously inoculated at 3 days of age with untreated and treated cultures*

Chicks (number)	Inoculated with culture—	Ration No.	Mortality in 21 days	Average weight of survivors at 21 days
			Percent	*Grams*
52	Untreated_____	391_____	53.8	112
49	_____do _____	391_____	75.5	107
101	_____	_____:_____	64.4	110
52	Treated [1]_____	391_____	98.1	94
50	_____do [2]_____	391_____	92.0	85
102	_____	_____	95.1	8
52	Untreated_____	391 +0.5 percent Ssm_____	36.5	120
50	_____do_____	_____do_____	54.0	117
102	_____	_____	45.1	118 8
51	Treated [1]_____	391 +0.5 percent Ssm_____	60.8	107
49	_____do [2]_____	_____do_____	57.1	96
100	_____	_____	59.0	101.4

[1] Grown in medium with 200 mg. of sodium sulfamerazine per 100 ml. for 268 days.
[2] Grown in medium with 200 mg. of sodium sulfamerazine per 100 ml. for 275 days.

chicks inoculated with the culture which had been grown in the presence of sodium sulfamerazine regardless of whether the chicks were given sodium sulfamerazine in the feed.

In a fourth series of tests made 23 days after the third test, but using ration 483, similar results were obtained among chicks not protected by sodium sulfamerazine. With the untreated culture the mortality was 32.4 percent and with the treated culture it was 61.8 percent (table 3). This difference in mortality is significant ($P<0.01$). These mortalities were lower than those on ration 391, which were 64.4 percent and 95.1 percent for untreated and treated cultures, respectively (table 2). The chicks referred to in table 2 were inocu-

TABLE 3.—*Mortality of chicks orally inoculated at 1 day of age with untreated and treated [1] cultures*

Chicks (number)	Inoculated with culture—	Ration No.	Mortality in 21 days	Average weight of survivors at 21 days
			Percent	*Grams*
51	Untreated_____	483_____	23.5	124
51	_____do_____	483_____	41.2	128
102	_____	_____	32.4	125.7
51	Treated_____	483_____	66.7	110
51	_____do_____	483_____	56.9	99
102	_____	_____	61.8	103.8
51	Untreated_____	483+0.5 percent Ssm_____	3.9	145
51	_____do_____	_____do_____	11.8	143
102	_____	_____	7.8	144
49	Treated_____	483+0.5 percent Ssm_____	10.2	139
49	_____do_____	_____do_____	4.1	129
98	_____	_____	7.1	133.3

[1] Grown in medium with 200 mg. of sodium sulfamerazine per 100 ml. for 298 days.

lated subcutaneously and those referred to in table 3 were inoculated orally. It has been found by Severens, Roberts, and Card,[5] that mortality among chicks subcutaneously inoculated at 3 days of age does not differ greatly from the mortality from oral inoculation at 1 day of age. The differences in mortality were about the same, 30.7 percent on ration 391 and 29.4 percent on ration 483. In the last test when the inoculated chicks on ration 483 were protected by 0.5 percent sodium sulfamerazine in the feed mortality was practically the same, being 7.8 percent for the untreated culture and 7.1 percent for the treated culture. A suggested explanation for this is that the combined protection of ration 483 and the sodium sulfamerazine was sufficient to reduce mortality to a very low level. Tests are now under way to determine the effect of several different rations on mortality among chicks inoculated with *Salmonella pullorum*. That the kind of ration has a marked effect on mortality from this disease has been reported by Roberts, Severens, and Card.[6]

In all the tests without sodium sulfamerazine in the feed the mortality among chicks inoculated with the treated culture was significantly greater than that among chicks inoculated with the untreated culture. This suggests a greater virulence of the treated culture. In all tests, except the first, when the treated culture had been grown in the 200-mg. concentration for only 36 days, the weights of the surviving chicks at 21 days were less for chicks inoculated with the treated than for those inoculated with the untreated culture whether or not sodium sulfamerazine was in the feed. This is also suggestive of a greater virulence of the treated culture. The average weight of all surviving chicks inoculated with the untreated culture is 128.8 gm. and 118.0 gm. for those inoculated with the treated culture. The difference is 10.8 gm. with a standard error of 2.94. The odds against this occurring by chance are more than 15,000 to 1.

The greater virulence of the treated culture in the later tests (tables 2 and 3) could be the result of a decreased virulence of the culture grown in normal medium without sodium sulfamerazine or to an increased virulence of the culture grown in the presence of sodium sulfamerazine. Whatever the explanation may be the results clearly indicate a greater virulence of the treated culture.

SUMMARY

Salmonella pullorum was grown in a medium containing sodium sulfamerazine. The medium with 200 mg. of sodium sulfamerazine per 100 ml. was markedly inhibitory to the growth of the untreated culture grown in normal medium. This could be interpreted as an acquisition of tolerance. However, the results indicate that the culture grown in the presence of sodium sulfamerazine for a long period of time was more virulent than the culture grown in the same kind of medium without sodium sulfamerazine when judged by mortality and weight of surviving chicks.

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VIVIPAROUS GROWTH IN IMMATURE BARLEY KERNELS[1]

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INTRODUCTION

That a cell or group of cells will continue to grow and multiply so long as the environment is suitable seems axiomatic. The embryo of a barley kernel developing in a spike enlarges rapidly for a time; then growth slows down and practically ceases. The endosperm continues to enlarge for a considerably longer time, until growth and starch deposition cease; then water loss and shrinkage of the seed to an air-dried condition follow. The seed remains relatively inactive until placed in an environment favorable for germination, when the embryo absorbs water and oxygen and resumes growth. In an earlier report (*12*)[2] it was implied that an inadequate supply of water to the embryo was responsible for the failure of the embryo to continue growth uninterruptedly to the seedling stage while still attached to the parent plant. The experiments reported herein were conducted to test that implication by comparing such viviparous growth with the normal development of the barley seed.

MATERIAL AND METHODS

Two pure lines of cultivated barley—one a six-rowed variety (Manchuria, C. I. 2330[3]; *Hordeum vulgare* L. emend. Lam.) and the other a two-rowed (Hannchen, C. I. 531, *H. distichon* L. emend. Lam.)—were grown in the greenhouse at the Plant Industry Station, Beltsville, Md., in the winter and spring of 1942. Flowers of each variety were emasculated and sib-selfed. A very good set of seed resulted in the Manchuria spikes, but because of poor pollen only a few Hannchen spikes were well filled. These were allowed to develop for 7 days and 9 days, respectively, when some of the spikes of each variety were treated to insure vivipary in some of their growing kernels by laying bare the embryo faces and providing them with a constant supply of water as previously described (*14*). The greenhouse temperature varied considerably, but a comparison with kernel growth obtained

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under constant temperatures (*13*) indicated that the growing temperature averaged about 21° to 22° C. Samples of five or more Manchuria kernels from both the untreated and the treated spikes of the same age after pollination were taken daily. Hannchen kernels were sampled every third day. The samples were killed and fixed in Craf fluid, embedded in paraffin, and sectioned sagittally 15μ thick. For the most part, Heidenhain's iron alum haematoxylin and Delafield's haematoxylin with safranine were used as stains.

A series of 5 to 8 typical but selected median sections was magnified approximately 10 times with a Leica enlarger and projected upon a sheet of 5 by 7 Kodabromide glossy paper. When this was developed as a negative print, developmental changes from day to day could be followed easily and quickly.

Since the observations on the Hannchen variety simply corroborated those with the Manchuria variety they are not reported herein.

For studying the development of starch in the kernel, sib-selfed Manchuria kernels sampled in January and February 1940 were used. Since the greenhouse temperatures then were considerably lower than when the 1942 material was grown, growth in the former was slower; but by comparing embryo sizes a series of samples that corresponded closely to the samples taken daily in 1942 was selected. The 1940 samples were sectioned in the same way as the 1942 ones, and the progress of starch development was studied by staining sections progressively and very lightly with very weak iron alum haematoxylin and then treating them with iodine-potassium-iodide solution.

OBSERVATIONS

As previously reported (*12, 14,*) vivipary can easily be induced in the growing barley kernel by supplying sufficient moisture to the face of its exposed embryo surface. Treatments given 6 to 9 days after pollination resulted in the appearance of seminal roots and the beginnings of plumule elongation on the fifteenth day after pollination. Probably because of differences in water absorption by different kernels, root and plumule emergence from the treated kernels were not simultaneous. The typical appearance of treated spikes was illustrated (*14, fig. 10*). Occasionally vivipary is induced in kernels wrapped in wet cotton without removing the lemma over the embryo. This indicates the availability of water as the chief factor in producing vivipary.

Studies of the development of the kernel of Manchuria barley at 20° C. have shown that 1 day after pollination the fertilized egg cell has divided once and the fusion nucleus has produced about 16 naked endosperm cells located at intervals in the undulating sheet of cytoplasm lining the egg sac (fig. 1, *A*). Four days after pollination (fig. 1, *B*) there are approximately 64 embryo cells and about 100,000 endosperm cells as estimated by extrapolation of the 20° curve (*13, fig. 2*). These endosperm cells form a continuous mass in the lower fifth of the embryo sac. Those adjacent to the embryo are the densest and most tightly packed. The remaining endosperm cells form a layer around the inside of the embryo sac, 2 or 3 cells thick on the chalazal side and only 1 cell thick on the opposite side. The distal ones are the last to get cell walls. By the fifth day after pollination (fig. 1,

C) the cavity within the embryo sac is filled with endosperm cells practically all of which have cell walls. The embryo is still undifferentiated on the sixth day after pollination (fig. 1, *D*). Meanwhile the ovule, which was about 1.6 mm. long on the first day after pollination, has grown to a length of about 5.4 mm.
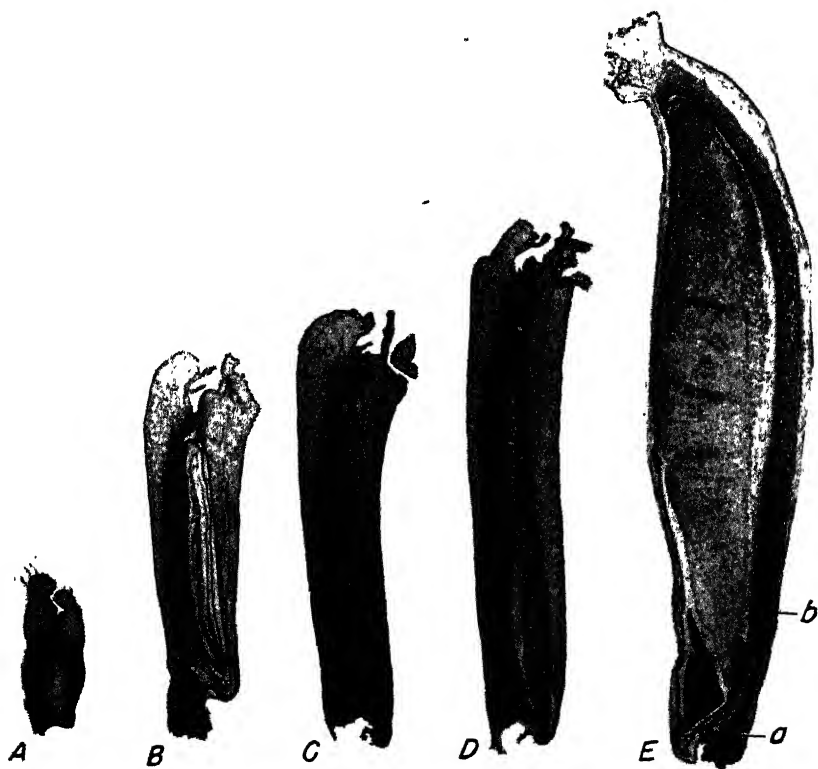
FIGURE 1.—Sagittal sections of barley ovules. *A*, One day after pollination, showing embryo sac containing antipodals, 2-celled embryo, and 6 of the 16 endosperm cells. *B*, Four days after pollination, when the endosperm cells extend full length of embryo sac and fill the lower fifth of it. *C*, Five days after pollination, when endosperm cells fill the embryo sac. *D*, Six days after pollination, when differentiation has just begun in the embryo. *E*, Eight days after pollination, showing fibrovascular bundle in the lower end of the furrow at the right; about 250 vessels are present at the level of the lower end of the embryo (*a*) and only about 25 at the level of the upper tip of the scutellum (*b*). All × 13.

On the day of treatment the Manchuria ovules have attained a length of approximately 7 mm. and the embryos an average length of 0.56 mm., which is equivalent to about 8 days' growth at 20° C. The plumule and primary root have begun to show differentiation; thereafter both treated and untreated kernels grew rapidly. The outlines of approximately median sagittal sections of embryos of Manchuria barley, beginning on the day of treatment for vivipary and at daily intervals thereafter, are shown in figure 2. The treated embryos
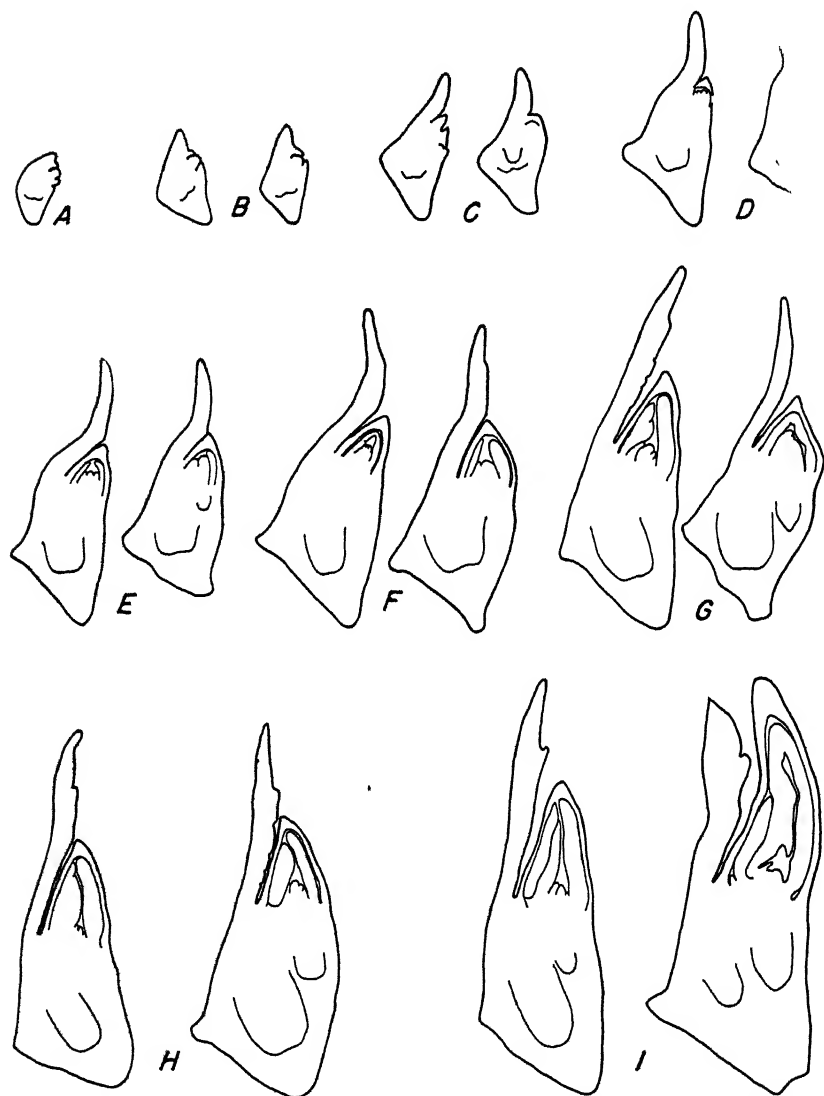
FIGURE 2.—Outlines, drawn with camera lucida, of median sagittal sections of barley embryos, 7 to 15 days after pollination. *A*, Seven days after pollination, the time of beginning of treatment to produce vivipary. *B* to *I*, Successive daily stages of embryos, untreated at the left and treated at the right; vivipary evident in *I* (right) 15 days after pollination. All × 20.

from 10- to 13-day samples are slightly smaller than those untreated, probably because of a slightly lower growing temperature brought about by evaporation of water from the treated spikes. The embryos in the untreated spikes apparently begin to lose moisture about 11 days after pollination, since the abscutellar surfaces of the embryos are noticeably less convex and turgid than those in the treated sam-

ples.   As it is not certain that any of the figures show the exactly median section, the apparent internal differences are of little significance.   The dimensions of the embryo outlines (fig. 2), however, correspond rather closely to the average size of embryos in approximately five kernels in each sample.   These dimensions, as plotted in figure 3, are the total length of the embryo and the length of the growing axis. The latter is the distance between the extreme distal extension of any part of the plumule and the end of the coleorhiza, not including seminal roots.   The total length of the normal, untreated embryo exceeds that of the treated from the eighth to the fifteenth day.   The
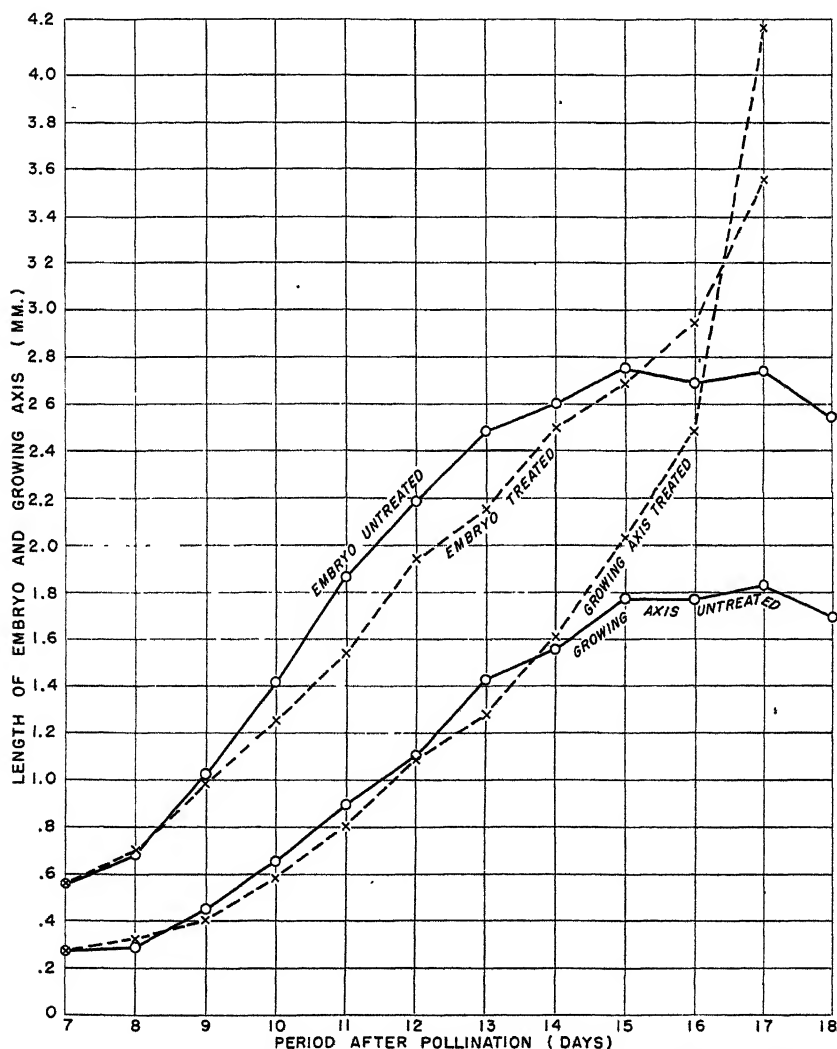
FIGURE 3.—Curves showing growth in length of embryo and of growing axis in untreated barley kernels and in those treated to produce vivipary.

curve of growth of the untreated embryo begins to be inflected on about the thirteenth day and flattens off sharply at 15 days. On the other hand, the growth of the treated embryo is rapid and continuous as shown by the uniform and steep slope of the growth curve. Similarly, the growing axis of the untreated check is longer than that of the treated embryo from the ninth to the thirteenth day; on the thirteenth day inflection begins in the untreated check and becomes complete on the fifteenth day after pollination. The treated embryo shows a very steady and rapid growth from the ninth to the seventeenth day, when the last samples were taken. The length of the growing axis overtakes that of the embryo on the sixteenth day. It is clearly evident from these curves that there is no dormant period in the development of embryos that have been successfully supplied with water. This conforms to the statement of Eyster (*3, p. 576*):

So far as observations have been made, viviparous maize plants have a continuous development from the fertilized egg to the mature plant so that dormancy and germination are not involved.

The seminal roots in each kernel of both untreated and treated samples were counted "in the section" and averaged for the sample (fig. 4). The average number of seminal roots for this variety (Man-
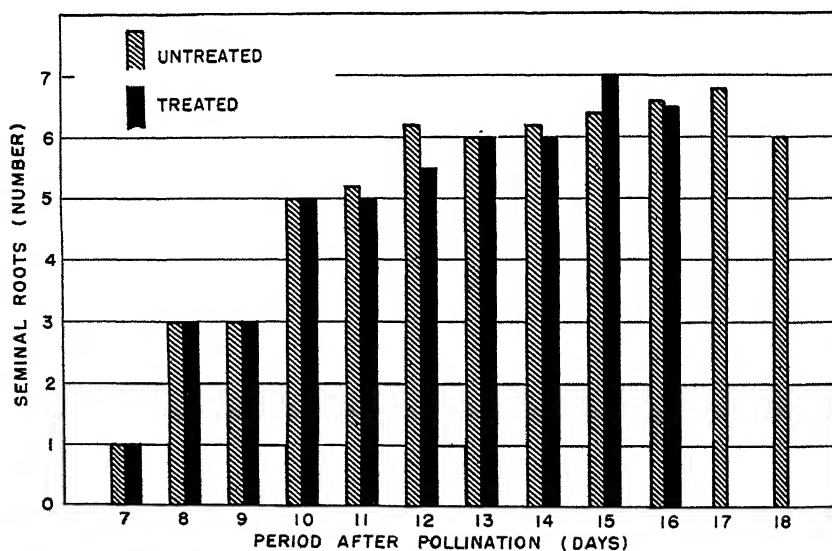
FIGURE 4.—Comparison of number of seminal roots in untreated embryos of barley and in those treated to produce vivipary.

churia) is 6.8 (*15*). Since the number of individuals examined in each sample rarely exceeded 5, no significant difference in root number is shown between normally growing and viviparous embryos. If the number of seminal roots is used as a criterion, embryo development may be considered at its maximum for the normal seed at 15 days after pollination. The normal embryo bears 5 to 11 seminal roots and root primordia, depending upon the variety (*15*).

It is interesting to note that an immature ovule after being harvested and dried will germinate immediately without either growing larger or becoming more fully differentiated. As shown previously (*15*), the seed may be considered viable whenever three seminal roots are demonstrable. From figure 2 it is seen that roots and plumule do not emerge from the treated embryo until it has reached the maximum development of the untreated embryo, namely, at 15 days after pollination at 20° C. The failure of the treated ovule to grow into a seedling at an earlier stage, as does the embryo of a seed harvested when immature, may possibly be explained by the presence, in the treated ovule, of the "embryo factor" suggested by Kent and Brink (*8*).

The normal-sized viviparous embryo extends its plumule and seminal roots until the nutrients available in the kernel are exhausted. Further growth can be induced by supplying a nutrient solution to the absorbent cotton wrapped around the treated spike. One such viviparous seedling continued to grow until the culm had attained an over-all length of more than 50 cm., when the invasion of the cotton by micro-organisms precluded further growth.

Normally, water is supplied to the growing kernel from the fibrovascular system of the culm by a branch bundle passing through the pedicel of the kernel. At the level of the base of the embryo 8 days after pollination the fibrovascular bundle of the pedicel supplying the floret contains about 250 vessels, each having a diameter of 5μ to 10μ (fig. 1, *E, a*). At the higher level where the mature kernel is attached, the number of vessels in the bundle is much less, because many have diverged into the bundles of the glumes, lemma, and palet of the floret On entering the kernel, the bundle divides to form the 2 lateral, the dorsal, and the ventral, or furrow, bundles (*7*). The furrow bundle is by far the largest of the 4, and about 25 vessels are distinguishable in it in transverse sections at the level of the upper end of the embryo of the 8-day kernel (fig. 1, *E, b*). Proceeding distally in the kernel, the vessels decrease in number until the last terminates at about nine-tenths of the distance to the distal end of the embryo sac. The number of vessels in the ovule appears to remain constant after about 8 days' growth. Since there are no vessels passing directly to the embryo, all water and dissolved nutrients must reach the embryo through adjacent tissue, which in turn is supplied with materials carried in the vascular system. The young embryo, then, is at the most favorable location for obtaining its supply of water and nutrients. It grows rapidly and at the end of 15 days has reached its maximum size and differentiation, and, in the normally growing ovule, without extending the plumule or radicle. The endosperm continues to grow thereafter and about one-third of the mature air-dry weight of the seed is added after the fifteenth day after pollination.

Although in the present study vivipary was produced readily when water was supplied to the exposed embryo area, naturally occurring vivipary is apparently rare. In 1909 or 1910 A. G. Johnson, of the Bureau of Plant Industry, Soils, and Agricultural Engineering, examined a sample from a meadow of green, standing timothy in southern Indiana, the ovules in which had produced seedlings in the spike after a protracted wet spell. J. H. Martin, of the same Bureau, has told the writer that this is a common occurrence in sorghums in the

southern Great Plains. Unlike the small, freely borne and easily dried-out spike of barley, or the maize ear adequately protected from external moisture by the husks, the dense sorghum head is exposed to drenching by rain and in protracted wet, humid periods the embryos may be supplied with enough moisture from the atmosphere to continue growth into seedlings. On the other hand, citations of the frequently observed "sprouting of grain in the shock" are not germane. These are merely instances of the germination of seeds that usually have matured on the stalk and dried down still further after harvest before absorbing enough rain water to produce germination.

Manifestly the lack only of water causes the embryo to go into the resting stage, since the only requirement for growth that was lacking in the untreated embryo, but available to the viviparous embryo, was distilled water. This is true not only for the spring-type variety Manchuria but also for three winter sorts that had proved to be extremely dormant when mature (*16*). If water is prevented from reaching the embryo at any stage after it has produced three seminal roots, a seed rather than a viviparous seedling is produced. In normal development the percentage of moisture in both embryo and endosperm decreases, slowly at first and then more rapidly, and the mature seed is the result.

In describing the progress of starch deposition in the barley kernel, Johannsen (*7*) stated that in two-rowed barley starch grains first appear within the endosperm cells when the kernel is 6 to 7 mm. long (about the stage when treatment to induce vivipary was given in the present study). He reported that starch first appears in the oldest endosperm cells, that is, those within the flanks of the upper part of the kernel but below the tip and that the younger peripheral region fills later. He believed that the endosperm cells can divide only until starch begins to be deposited in them.

In figures 5 and 6 are shown near-median sagittal sections of Manchuria barley kernels growing normally at 21°+ C. from the second to the fifteenth day after pollination. Development in them has progressed slightly further than in those of the same age grown at a constant temperature of 20° (fig. 1). Iodine-potassium-iodide solution stains mature starch grains deeply and younger grains less so or not at all. Very young, unstained starch granules may often be detected in the cells at higher magnifications.

Starch is clearly seen in the ovary wall at the distal end of the ovule on the second day after pollination (fig. 5, *A*). At higher magnifications starch grains progressively decreasing in size and age downward on the furrow (ventral) side, with a few very young grains scattered through the cells of the dorsal wall, were visible. By the third day (fig. 5, *B*), stainable grains appeared along the entire length of the furrow in the tissue surrounding the fibrovascular bundle and smaller, lightly staining grains were evident in the dorsal wall. On the fourth day (fig. 5, *C*), the dorsal wall had still more and larger starch grains and the furrow region had an abundance of starch. Up to the fourth day starch is apparent in maternal tissue only. The first appearance of starch in zygotic tissue occurred on the fifth day after pollination (fig. 5, *D*), when very small grains had appeared in the distal end of the endosperm mass, which by that time entirely filled the cavity of
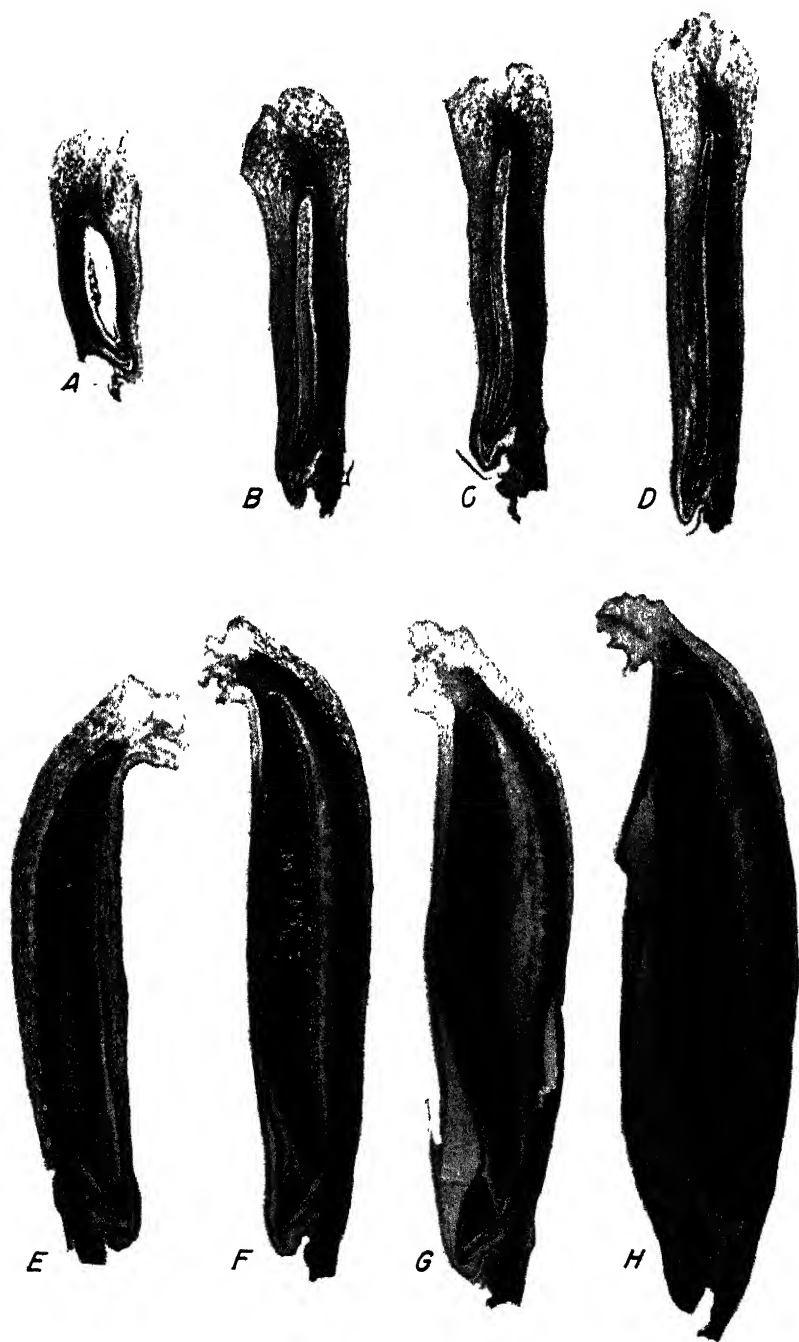
FIGURE 5.—*A* to *H*, Successive daily near-median sagittal sections of untreated barley kernels showing position and extent of starch deposit from the second to the ninth day after pollination (stained with iodine-potassium-iodide solution). × 13.
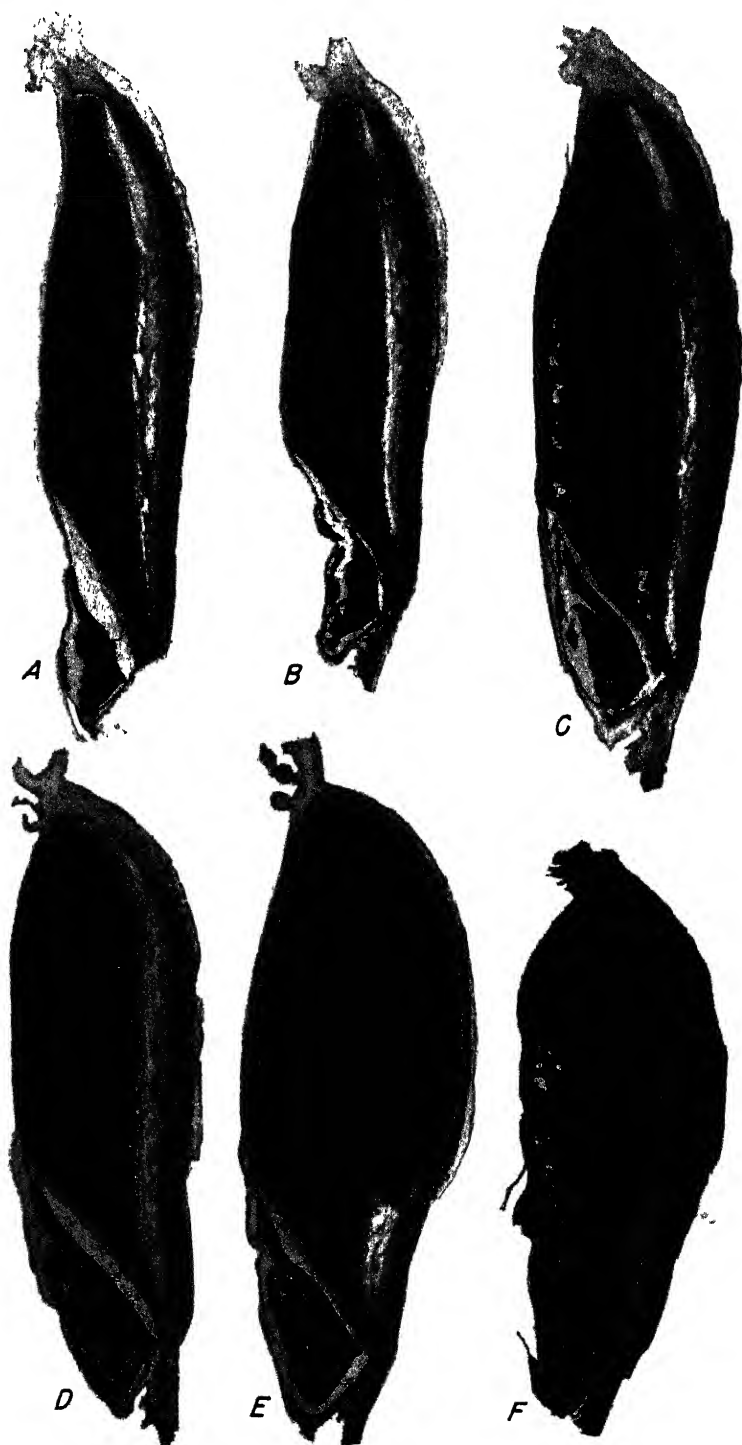
the embryo sac. From that time on, endosperm starch increased rapidly in amount and density. On the sixth day (fig. 5, *E*) there were a few small grains in the basal third of the embryo. Starch was more abundant in the embryo on the seventh day (fig. 5, *F*) but was still confined to the lowest third; but by the eighth day it had appeared in the coleoptile and in the upper tip of the scutellum (fig. 5, *G*). In later stages (figs. 5, *H*, and 6), the ground tissue of the embryo was well supplied with stored starch, ideally located for ready utilization during germination. The formation of dense starch in the endosperm had not yet progressed downward to the level of the embryo until the eighth day (fig. 5, *G*), but by the ninth day (fig. 5, *H*) it had packed the cells to almost the proximal tip of the endosperm mass. The natural position of the embryo with the scutellum rather closely applied to the endosperm (fig. 6, *F*) was accidentally shifted in this and several other sections (fig. 6, *A*, *C–E*) during the killing, fixing, embedding, and sectioning of the kernel. In these near-median sections, the endosperm next to the embryo is thinner than it is in either flank, chiefly because of the keeled surface of the scutellum.

As has been stated, the endosperm cells had completely filled the cavity of the embryo sac and 5 days after pollination they were actively growing and multiplying. Two days later, when the treatment to induce vivipary was given, the moisture content of the ovule was approximately 80 percent (*6*) and the ovule had an oven-dry weight of about 2 mg. As shown in figures 5 and 6, the 8 days between treatment and appearance of vivipary is a period of extremely rapid growth and deposition of starch in the endosperm. This starch deposit is the main cause of increase in lateral and dorsiventral diameter and of decrease in moisture content up to the end of kernel growth. Fifteen days after pollination, when vivipary is evident in treated spikes, the dry weight of normally growing untreated ovules had increased to about 23 mg. while the water content had dropped to only about 60 percent. The air-dry weight of mature greenhouse seeds of Manchuria barley at 12 to 13 percent moisture is about 38 mg. per kernel. If a moisture content of 12 percent is assumed, the dry weight would be 33+ mg.

During early development the zygote lies in a lake of cell sap, which supplies it unstintedly with water and nutrients. The formation of endosperm cells under the scutellum tends to impede the passage of nutrients by osmosis from the furrow bundle, but enough still gets through to permit maximum growth of the ovule until inflection of the embryo growth curve on the thirteenth day after pollination. As kernel development progressed, the deposit of starch pushed the absorbing scutellum farther and farther away from the source of nutrient supply in the vascular bundle of the furrow. Only its lower end remained approximately in its former position. At the beginning of inflection of embryo growth on the thirteenth day (figs. 3 and 6,

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EXPLANATORY LEGEND FOR FIGURE 6

A to *F*, Successive daily near-median sagittal sections of untreated barley kernels showing position and extent of starch deposit from the tenth to the fifteenth [day after pollination (stained with iodine-potassium-iodide solution). × 13.

D), only this lower tip and the lower end of the ventral side of the embryo were readily accessible to the furrow, and these were being dehydrated. The furrow region was the last to remain green, and starch continued to be laid down in the young endosperm cells adjacent to it until desiccation of the kernel stopped all growth. This increasing interposition of gradually dehydrating starch-filled endosperm cells between the scutellum of the embryo and the source of supply of water and nutrients in the furrow bundle appears to be a sufficient cause for the inflection of the growth curve of the untreated embryo on the thirteenth day after pollination (fig. 3).

DISCUSSION

According to Gray (*5, p. 882*) a seed is "the ripened ovule, consisting of the embryo and its proper coats." Gager (*4, p. 453*) said that with few exceptions "the distinctive features of a seed is a resting embryo." The stage of maturity at which drying down may occur without sacrificing viability may vary considerably. Barley kernels taken from the spike as early as 9 or 10 days after pollination will, on drying, become "seeds" that are able to germinate and produce plants that later develop mature seed. Sections of embryos of these very young seeds show a very short plumule with the beginnings of about 3 leaves and the rudiments of 3 seminal roots (*15*). On the other hand, under good growing conditions embryos of barley ovules left to mature on the plant will attain a much greater size, with well-developed plumules and the normal complement of seminal roots, which may total as many as 10 or 11 in individual seeds of certain varieties.[4] Recently Kent and Brink (*8*), in growing excised immature barley embryos on sterile media, found that, while such embryos normally germinate within 2 or 3 days and become very small, spindling seedlings, the addition of certain substances, such as water extracts of dates and bananas, milk, and tomato juice, will delay germination and allow the embryos to grow and develop *as embryos* until they reach or surpass the size of embryos matured on the plant. Such an "embryo factor," present in normally growing barley kernels but absent in excised immature kernels, should account for the absence of further pregermination development in the embryos of immature barley seeds.

The embryo of the untreated normally maturing seed loses water. The insufficient water supply precludes starch digestion and nutrient transfer, and the embryo goes into the resting condition. A resting stage may be forced upon an immature embryo by drying, or the seed may attain "maturity" through natural desiccation. On the other hand, if water is supplied constantly to the developing embryo, the embryo not only attains the maximum size and development of that in the normally matured seed in about 14 days from pollination but it continues to grow thereafter and form the seedling, with no inflection of the growth curve. In the viviparous seedling the conditions of youth are largely maintained; there are ample nutrients available in the endosperm adjacent to the scutellum, starch-digesting enzymes

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[4] In 1943 when barley seeds were germinated in petri dishes 1 seed of the variety Alpha produced 11 seminal roots and 1 of the variety Primus 10.

are present in the secretory layer of the scutellum, and sufficient water reaches all parts of the embryo to provide for uninterrupted growth. Such a seedling, if pricked off, is able to produce a plant that yields normal seed.

In a viviparous seedling, the course of normal growth as occurring in juvenile plants or plant parts obtains and growth proceeds upon the compound-interest principle. The cell walls are soft and permeable, and there is no mechanical limitation to cell division. Successive increments are added to the "principle" of reactive material, but, as has been noted (*11*), growth rate is conditioned by nutritional opportunities. As strengthening, supporting, and nutrient materials are laid down in the growing entity, nutrition is impeded by desiccation, cell growth and division are retarded, and the grand period of growth suffers its inflection. Carried still further, these aging factors result in dormancy or death.

The conception of vivipary herein presented gets considerable support from the findings of Eyster (*3*), who stressed the fact that vivipary is a fundamental and primitive character and cited its occurrence in all great divisions of the plant kingdom (*3*). He attributed the dormancy of the embryo of the mature seed to insufficient water supply and stated that if developing maize ears are kept under suitable conditions of moisture the sporophytes appear to have a continuous growth from the fertilized egg to the new plant (*1*). Moreover, as in normal immature barley ovules, he found that viviparous kernels of maize that had been forced into dormancy by artificial drying germinated when they were exposed to suitable growth conditions (*3*).

A large proportion of the viviparous kernels found in maize possess "deficient" endosperms. Eyster (*2, p. 250*) stated:

. . . practically all of the kernels with reduced endosperm have viviparous embryos due to the very close linkage between re_2 vp_2.

Naturally, a reduced or deficient endosperm would tend to make less effective the starch endosperm block to the passage of water from the fibrovascular system to the embryo. This would facilitate vivipary.

Mangelsdorf (*10, p. 493*), however, attributed the phenomenon of vivipary to the action of genes, citing "fifteen different genetic factors and nine distinct characters" involved in its inheritance. He said (*10, p. 490*):

Dormancy of the seed during development is so general that it may almost be taken for granted. Yet we find that at least fifteen different genetic factors are involved in maintaining dormancy and we can scarcely suppose that this is more than a sample of the total number.

He (*9*) used the stage of kernel development at which vivipary occurs as a basis for classifying his different types of the phenomenon. On the other hand, Eyster (*3*) found different stages of vivipary in the same ear of maize. Similarly, it was found in the present study that kernels on the same barley spike showed different stages of root and plumule emergence.

To the writer, Mangelsdorf's interpretation seems a greatly exaggerated complexification of a very simple phenomenon. Vivipary is the *natural* course, and probably there are many ways whereby the normal desiccation of the growing embryo may be prevented, any one

of which will allow vivipary. It seems probable that the so-called vivipary genes reported by Mangelsdorf (*10*) function by varying the structure of the kernel; this variation in turn may restrict the quantity of water available to the growing embryo.

If, as Eyster (*3*) believed, the typical resting condition of seeds is an adaptation forced on the organism to insure survival, vivipary should occasion no surprise. The phenomenon that demands explanation is the *dormancy* of the mature seed. To the writer it seems clear that the maturation of the seed is brought about by structural factors that inhibit growth of the embryo by gradually cutting off its supply of water. In barley, the most likely block to water passage consists of the starch-packed endosperm cells massed between the embryo and the fibrovascular bundles lying in the still chlorophyllous tissue of the furrow.

SUMMARY AND CONCLUSIONS

Viable resting embryos may be induced in barley by either drought or harvest at any time after their earliest viability stage when three seminal roots are present. Their development into seedlings is proof that such seeds have sufficient stored nutrients except water and oxygen.

Under optimum natural growing conditions the embryo becomes dormant after reaching the maximum size and development that is possible under the conditions of gradual desiccation in the maturing seed.

When distilled water is supplied to the abscutellar surface of the growing embryo, growth is continuous and vivipary occurs.

Ovules treated on the seventh day after pollination to induce vivipary developed similarly to normally growing ones in all observed features up to the thirteenth day, except that after the ninth day the embryo appeared to be more plump or turgid in the treated ovule than in the untreated one. Growth of the plumule was first visible in the viviparous ovule on the fourteenth day. Seminal roots had emerged and the plumule was actively growing on the fifteenth day after pollination.

There was no significant difference in the number of seminal roots on the viviparous and the normally matured embryos.

In a normally growing ovule a resting stage is forced upon the growing embryo, while in the ovule treated to induce vivipary no such interruption to growth, or even lessened growth rate, occurs.

In the normally growing ovule water adequate for vivipary is blocked off probably by the interposition of a dense layer of starch-bearing endosperm cells between the embryo and the fibrovascular bundle in the furrow.

The thesis is submitted that vivipary, rather than itself being a genetic character, is merely the result of any condition, character, or seed deficiency that will allow the access of water to the embryo.

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TIP BLIGHT OF GARDEN PEA [1]

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INTRODUCTION

In 1941, plants of garden pea (*Pisum sativum* L.) with dead tips were noted in a seed-treatment experiment near Madison, Wis. Plating of diseased tissue on agar yielded a species of *Pythium* which upon inoculation of potted plants in the greenhouse produced similar symptoms. Correspondence with Dr. W. T. Schroeder, New York Agricultural Experiment Station, revealed that he had noticed the disease in the same locality in 1940 and had also isolated *Pythium*, although no controlled inoculations were made. The disease was seen in commercial fields of canning peas in 1942 and 1943. Damage was generally distributed, but slight, with the exception of one field in 1942 where 40 percent of the plants had killed tips.

In 1946, in a nursery near Madison, an average of 6 percent of the plants and 10 to 15 percent of some lots had killed tips. This amounted to considerable damage since most of the lots were single-plant progenies planted for further selection. The disease was observed in several commercial fields during that season, one of which had 20 percent of the plants diseased in a 2-acre section of the field. Apparently the same species of *Pythium* was isolated in all cases from diseased plants and it was tentatively identified as *Pythium ultimum* Trow. Because of the unusual nature of the disease a more detailed investigation was made, a preliminary report of which was published in 1947 (*4*).[2]

Pythium ultimum was described by Trow (*13*) in 1901 as a saprophyte. Since that time it has been reported many times as a parasite. Middleton in 1943 (*8*) listed 148 plants as hosts and noted that it was perhaps the most common member of the genus in the United States and that it was found frequently in other countries. It has been reported frequently in association with damping-off and with root rot of peas (*1, 10, 11*). No record has been found of this, or any other species of *Pythium*, occurring on the above-ground foliage of the pea plant independently of infection originating below the soil level.

Middleton (*7*) in 1941 described a rot of roots, crowns, buds, and petioles of rhubarb incited by *Pythium anandrum* Drechsler, *P. oligandrum* Drechsler, and *P. ultimum*. Middleton et al. (*9*), in 1942 de-

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scribed a stem and lower-leaf rot of fibrous-rooted begonia incited by *P. debaryanum* Hesse, *P. splendens* Braun, and *P. ultimum*. *P. ultimum* was the most pathogenic on rhubarb and the most frequent on begonia. In both cases rot of the aerial plant parts followed infection of the lower parts. Tompkins et al. *(12)* in 1939 found *P. ultimum* inciting a soft rot of the fruits of Zucchini and Mammoth Summer Crookneck pumpkin and of watermelon. Fruits of tomato, bean, eggplant, and field pumpkin became infected when inoculated without wounding and several other types of fruit tested became infected when inoculated through wounds.

Similar diseases of fruits, storage organs, and foliage parts (infected by progression of the pathogen from tissue at the soil level or below) have been reported as incited by still other species of *Pythium*. Drechsler *(2)* in 1925 found cabbage heads rotted by a *P. debaryanum* type with the fungus spreading out from the fleshy core and leaf bases to the lamella. A similar rot was produced when inoculation was made with several other species of *Pythium;* some species were not pathogenic. Harter and Zaumeyer *(5)* in 1931 reported a bean wilt, due to *P. aphanidermatum* (Edson) Fitz. (*P. butleri* Subr.), which started at the ground line and progressed up the lower stem to the lower branches. Wound inoculation at the cotyledonary node resulted in infection.

Elliott *(3)* in 1943 described a stalk rot of the lower nodes of corn incited by *P. aphanidermatum* (*P. butleri*) and reproduced the disease in the field and greenhouse. The crown and roots were not involved when the disease was encountered in nature and the roots were not infected when inoculated in the greenhouse. The activity of the fungus in the stalks ceased abruptly and demarcation of invaded tissues was strong. Wallace *(14)* in 1944 reported a species of *Pythium* or *Phytophthora* which incited damping-off and foot rot of papaw in East Africa but stated that most infections occurred through leaf and fruit scars on the upper part of the plants, spreading to stems, leaves, and fruits. These two are the only reports found of independent infection of aerial parts of plants, other than fruits or storage organs, by species of *Pythium* and in one of them the identity of the fungus is uncertain.

EXPERIMENTAL RESULTS

SYMPTOMS

Infection takes place in the bud or the axil of a young leaf. In the field, affected tissues are at first water-soaked with a fading of the green color. This stage does not last long, for invaded tissues are killed very rapidly. As desiccation ensues the tissue shrivels and the color changes to straw color to black (fig. 1, *A*) depending on conditions during drying. The fungus advances downward from the tip, rarely killing more than three to five internodes. Girdling takes place from the leaf axil with collapse of that node and consequent killing of the tip above it (fig. 2). Occasionally, girdling is incomplete with the result that a part or all of the leaf is killed and the tip of the plant survives. In exceptional cases one or more leaflets may be partially or entirely killed as a result of local infection rather than from that originating in a bud or leaf axil, while sometimes the entire plant, particularly if it is quite young, may be killed.

FIGURE 1.—Pea plants from nursery near Madison, Wis., showing effects of tip blight due to *Pythium ultimum*: *A*, Growing point and two nodes below it killed; *B*, later stage with lateral shoots started below blighted tip.

When a tip or leaf of a plant is killed, progress of the lesion halts abruptly and the line of demarcation at the junction of healthy and diseased tissue is sharp. The fungus is not visible macroscopically on the exterior of diseased parts, but often a cottony web of mycelium containing fruiting bodies appears in the hollow of the stems. Diseased plants have been found ranging in maturity from those just emerged to those in blossom. However, in two adjacent lots differing in size, a greater percentage of infected plants is generally found in the shorter plants.

As soon as the tip of a plant is killed new shoots are put out in the axils of lower leaves (fig. 1, *B*). If the plant is young, one shoot quickly gains ascendency over the others and the affected plant may be overlooked later in the season unless the later maturity is noted. On larger plants there is more competition among the new shoots.

FIGURE 2.—Pea plant from nursery near Madison, Wis., infected in leaf axil by *Pythium ultimum.* Top is beginning to wilt and lateral shoots have started.

All of them may grow for some time and as many as five of the upper ones may continue to be about equal in vigor.

Symptoms identical with those found in the field were produced by suitable artificial inoculation of young potted plants in the greenhouse (fig. 3, *A*). The water-soaked appearance of the tissues was evident within 48 hours and subsequent drying and discoloration were very rapid. When left on the greenhouse bench after the original treatment, the plants put forth new shoots from the axils of lower leaves. A type of symptom not seen in the field was produced in the greenhouse by spraying the entire plant with a suspension of mycelial fragments of the fungus. With 6 to 24 hours in a moist chamber, there were scattered over the leaves and stems many small water-soaked spots that dried to a very light brownish tint. If left in the moist chamber for 48 hours all plants were completely killed. In bud inoculations, when the tip escaped killing, one or more leaves or parts of leaves were frequently killed.

FIGURE 3.—*A*, Delwiche Commando pea plants inoculated in the terminal bud with *Pythium ultimum* and kept in moist chamber for 48 hours; note lateral shoots at bases of plants. *B*, Uninoculated.

THE CAUSAL ORGANISM

Isolations made from diseased plants when the disease was first noted in 1941 consistently yielded a fungus identified as a member of the genus *Pythium*. All isolations were from plants from the same plot. No effort was made to determine the species. In 1946, numerous isolations were made from diseased plants in the nursery at Madison and from material collected in two widely separated commercial plantings in Wisconsin. These consistently gave the same species and it was apparently similar to the one isolated in 1941, although no cultures made in 1941 were available for comparison. Collections from two commercial plantings in 1947 gave the same species upon isolation.

One of the isolates made from a plant in the Madison nursery in 1946 was selected as typical and a detailed examination was made. It was fast-growing, producing an abundance of white, cottony mycelium on potato-dextrose agar, and numerous oospores and

sporangia on the surface of the agar within 10 days to 2 weeks. It grew well on a variety of media with variation between media in the amount of aerial mycelium and fruiting bodies produced. Fruiting, both sexual and asexual, was best when a small block was cut from a vigorous culture on potato-dextrose agar and placed in distilled water. Germination of oospores was not observed. Germination of sporangia was by germ tubes only.

Spore sizes, sexual apparatus, germination of the sporangia, and growth habits of the fungus all agreed with the description of *Pythium ultimum* Trow. (*13*) as given in Middleton's monograph (*8*).[3]

PATHOGENICITY

When a species of *Pythium* was isolated from diseased pea plants in 1941 young pea plants of the Wisconsin Perfection variety about 5 inches tall were inoculated in the greenhouse by placing small agar blocks containing the fungus in the terminal buds of some plants and in the upper leaf axils of others. After inoculation the plants were kept in a moist chamber for 48 hours. All of the tips of the plants were killed regardless of the point of inoculation, and symptoms were similar to those seen in the field. The fungus was reisolated from some of the diseased plants. An equal number of uninoculated plants remained healthy.

Five isolates from material from the Madison nursery were used in preliminary inoculations in the greenhouse in 1946. There were no differences in type or degree of symptoms produced. Therefore, the isolate selected for detailed examination in the laboratory was used in all subsequent inoculations. Delwiche Commando and Wisconsin Perfection varieties of peas were used in the inoculation experiments. Since both varieties were very similar in their development and both were identical in reaction to the fungus, the variety has not been specified in reporting individual experiments. Except in temperature experiments, plants averaged 4 to 6 inches tall with four to seven leaves each when inoculated. All plants were grown in sterilized quartz sand in 6-inch clay pots and were watered on alternate days with a balanced nutrient solution.

Inoculation was tried first by placing agar blocks containing the fungus in buds of plants and in axils of young leaves. Considering only those left in the moist chamber for 48 hours, the tips were killed on each of 12 plants inoculated in the bud and on 15 of 16 inoculated in leaf axils. The fungus was recovered from all plants. Forty plants left uninoculated remained healthy. A second method of inoculation was to spray the entire plants with a suspension of mycelial fragments prepared by growing the fungus for 6 days on 100 cc. of a modification of Czapek's solution [4] in 250 cc. Erlenmeyer flasks at 28° C. and macerating the mycelium 30 seconds in a Waring Blendor. Of those left in the moist chamber 48 hours 17 of 18 plants were collapsed and dead 2 days after removal. The eighteenth plant

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[3] Appreciation is expressed to Dr. John T. Middleton, Citrus Experiment Station, Riverside, Calif., who kindly checked the culture sent to him and confirmed the identification.

[4] KNO_3, 3 gm.; KH_2PO_4, 1.0 gm.; $MgSO_4 \cdot 7H_2O$, 0.5 gm.; KCl, 0.5 gm.; $FeSO_4$, trace; dextrose, 30 gm.; water, 1,000 ml.

had two leaves alive at that time.   No attempts were made to recover
the fungus.   None of the controls were diseased.   A third means of
inoculation was to place a drop of the suspension described above in
the bud of the plant or in a selected leaf axil with a medicine dropper.
In a representative experiment, of those left in the moist chamber
for 48 hours, the tips were killed on 71 of 74 plants inoculated in the
bud and on 29 of 66 plants inoculated in leaf axils.   Twenty-three
of the plants without the tip killed had part or all of the leaf killed
at the node of inoculation.   Isolations were made from 20 diseased
plants of each type of inoculation and the fungus was recovered from
each of them.   Uninoculated plants in eight pots, averaging seven to
nine plants per pot, kept in the moist chamber 48 hours, remained
healthy.

Of the inoculations described only the plants that were left in the
moist chamber 48 hours are itemized.   Successful inoculations were
secured on many more plants in these particular series of inoculations
and in other series not discussed, although few other attempts were
made to reisolate the fungus.   Thus it was demonstrated that the
fungus can incite the disease seen in the field.

After the pathogen was identified as *Pythium ultimum*, a common
soil organism frequently associated with damping-off of peas and other
crops, it became of interest to examine its potentialities in damping-
off of peas in the greenhouse when mixed with the soil in pure culture.
Flats of unsterilized greenhouse compost soil were prepared and 300
cc. of the mycelial-fragment suspension was thoroughly mixed in
alternate flats.   All flats were left at 24° C. for 7 days and the soil
was kept moderately moist.   Six rows of 20 seeds each of Delwiche
Commando peas were planted in each flat and 1 inoculated and 1
check flat were placed at each of several temperatures.   The results
at 28° C. 19 days after planting may be seen in figure 4.   There were
111 seedlings in the check flat or an average of 18.5 per row, an aver-
age emergence of 92.5 percent healthy plants.   There were nine
seedlings in the inoculated flat not showing advanced stages of
damping-off, or an average emergence of 7.5 percent healthy plants.

Since the pathogen concerned was found to be an active damping-off
agent of peas and there are other species of *Pythium* that act likewise,
the question arose as to whether other species of the genus could cause
the same type of above-ground disease.   Another *Pythium* species,
tentatively identified as *P. debaryanum*, was obtained from M. P.
Backus, University of Wisconsin.   It was compared with *P. ultimum*
in a series of inoculations replicated twice at temperatures of 16°, 20°,
24°, and 28° C. and periods in the moist chamber of 24, 48, and 72
hours at each temperature.   Inoculation of both was by drops of
mycelial-fragment suspension, prepared as described above, in buds
of the plants.   The symptoms produced on the plants by *P. debary-
anum* were the same in type as those produced by *P. ultimum* but were
much less severe under all conditions tried.   A typical response may
be seen in figure 5, made 10 days after inoculation at 28° with 48 hours
in the moist chamber.   Here, where 100 percent of the plants had two
to three nodes killed by *P. ultimum*, only 60 percent of the tips were
killed by *P. debaryanum* and then only the uppermost one, or rarely
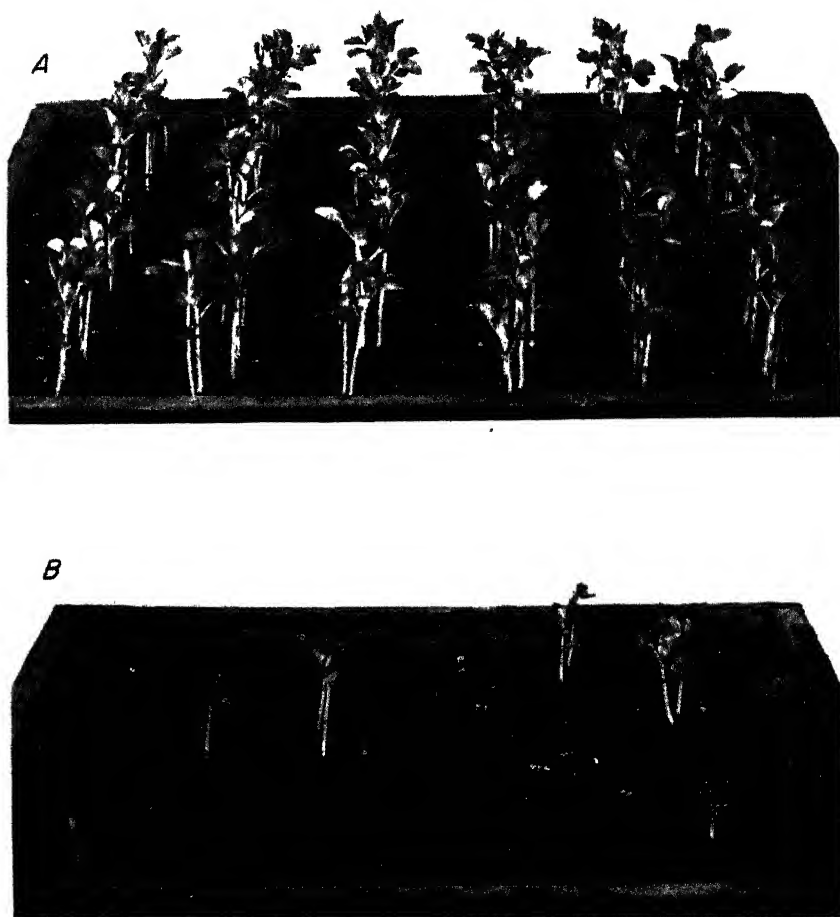
FIGURE 4.—Delwiche Commando peas 19 days after planting, 20 seeds per row, in greenhouse compost at 28° C.: *A*, Uninoculated; *B*, soil inoculated with *Pythium ultimum*.

two, nodes. No attempt was made to increase the virulence of *P. debaryanum* by repeated passages through the host.

EFFECT OF MOISTURE AND TEMPERATURE ON INFECTION

When inoculations were made by placing agar blocks in buds, percentage of infection and severity of the disease were increased by putting the plants in a moist chamber. However, over 50 percent of the tips of plants treated in this manner were killed with no exposure in the moist chamber. No infection resulted when agar blocks were placed in leaf axils unless the plants were left in the moist chamber at least 6 hours. When plants were sprayed with the mycelial-fragment

FIGURE 5.—Delwiche Commando peas grown in sterilized quartz sand at 28° C.: *A,* Uninoculated; *B,* inoculated in terminal bud with *Pythium debaryanum; C,* inoculated in terminal bud with *Pythium ultimum.* Plants placed in moist chamber for 48 hours after inoculation.

suspension, small spots or edges of the leaf tissue were killed with no moist-chamber treatment. These methods were not considered representative of what might be expected to happen in the field.

The inoculation treatment in which drops of mycelial-fragment suspension were placed in buds or leaf axils probably most nearly approached infection under field conditions. When this method was used, a period of 24 to 48 hours in the moist chamber was necessary for infection to take place. At higher temperatures (24° and 28° C.) the time required was shorter, as shown in table 1. Usually most of the tips were killed at these temperatures with an exposure of 24 hours, whereas at 16° it took over 72 hours in the moist chamber after inoculation to approach this level of disease. In all cases the percentage of infection was higher and the disease was more severe when the drops were placed in the bud.

TABLE 1.—*Effect of length and temperature of the inoculation period on tip blight following inoculation in the bud with Pythium ultimum*

Hours in moist chamber	Percentage of plants with tips killed at—			
	16° C.	20° C.	24° C.	28° C.
24	0	11. 6	93. 3	100. 0
48	6. 2	80. 8	100. 0	100. 0
72	77. 0	93. 3	100. 0	100. 0

It was noted in the field and in the greenhouse that the progress of the fungus stopped as soon as the plants were in relatively dry air. Apparently only that tissue invaded when a high relative humidity prevailed was involved. Plants staked in the field after the initial observation rarely showed any extension of lesions and those left on

the greenhouse bench after tips were killed remained healthy below the original lesion. Six pots of such plants, averaging eight plants per pot, were replaced in the moist chamber 3 weeks after inoculation. They were removed at 2-day intervals. There was some further killing of stems and leaves of 80 to 90 percent of the plants removed after 2 days and 100 percent killing of the entire plant of those left 6 days.

Temperature had a marked effect on infection and development of the disease. Experiments were conducted in constant-temperature houses held at 16°, 20°, 24°, and 28° C. Moist chambers in which plants were placed after inoculation were in each of the houses. There was a reduction in temperature in the moist chambers due to the cooling effect of evaporation, temperatures there being about 13°, 17°, 21°, and 25°, respectively. Since the effect of the reduction in temperature during the inoculation period compared to that of the post-inoculation period was not determined, results are given on the basis of the temperatures of the latter period.

In all cases there was an increase in percentage of infection or severity of disease or in both from 16° to 24° C., with very little or no differences between 24° and 28°. The effects of temperature and length of time in the moist chamber were interrelated, for with a longer time in the moist chamber the percentage of infected plants at the lower temperatures increased. However, if plants at all temperatures were left long enough for infection to approach 100 percent at 16°, the disease involved more nodes as the temperature increased.

To determine the effect of pretreatment temperature on the reaction of the plants to the disease, plants grown at 16°, 20°, 24°, and 28° C. were distributed at each of the four temperatures immediately prior to inoculation. The plants were 18 days old and varied in size from 2 inches high with four nodes at 16° to 6 inches high with seven nodes at 28°. There were 5 pots with 8 to 10 plants each in each treatment. Two were left as checks and three were inoculated. Inoculations were made by placing a drop of mycelial-fragment suspension in the bud of each plant and the plants were left in the moist chambers 68 hours. Ten days after inoculation plants were rated in four classes as follows: (1) Tips dead; (2) one or more leaves completely killed; (3) no leaf entirely killed but lesions on leaflets or petioles; (4) healthy. A disease index was calculated for the plants in each pot [5] and the indices for the three pots in each treatment averaged. The results may be seen in table 2. The influence of temperature after inoculation was highly significant and there was a definite increase in disease index from 16° to 24°. The length of time in the moist chamber may have obscured some of the differences at the higher temperatures. The influence of preinoculation temperature was significant at the 5-percent level with a higher disease index at 20° than at 16° and 24°. It is doubtful if this relationship would be consistent in further trials.

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[5] Number of plants in classes 1, 2, 3, and 4 multiplied by 3, 2, 1, and 0, respectively; total divided by three times the total number of plants, and result multiplied by 100.

TABLE 2.—*Effect of temperature before and after inoculation on pea plants inoculated in the bud with a drop of mycelial-fragment suspension of Pythium ultimum*

[Averages of disease indices from 3 pots of 8 to 10 plants each in each treatment]

Preinoculation temperature (° C.)	Disease index at temperature after inoculation indicated				Average [1]
	16° C.	20° C.	24° C.	28° C.	
16	51.1	88.0	95.5	96.3	82.7
20	61.1	94.4	100.0	97.8	88.4
24	48.6	86.2	93.0	90.0	79.5
28	48.7	94.3	98.0	97.8	84.7
Average [2]	52.4	90.7	96.6	95.5	

[1] Differences significant.
[2] Differences highly significant.

MSD at 5-percent level: 5.4.

Seeds were planted in inoculated and uninoculated soil in flats. After planting, one flat of each was placed at 16°, 20°, 24°, and 28° C. Stand counts were made 26 days after planting and the stand in each of the six rows of each flat averaged. The results are shown in table 3. The percentage reduction in stand rose from 52.9 at 16° to 91.9 at 28°.

TABLE 3.—*Effect of temperature on stand of peas in uninoculated greenhouse compost in flats and in the same soil inoculated with Pythium ultimum*

[Average stand per row from 20 seeds in each of 6 rows per flat]

Treatment	Stand at temperature indicated				Average
	16° C.	20° C.	24° C.	28° C.	
Uninoculated	15.3	18.3	15.6	18.5	16.9
Inoculated	7.2	5.5	3.5	1.5	4.4
Percent stand reduction	52.9	70.0	77.6	91.9	74.0

Petri dishes containing 20 cc. of potato-dextrose agar were inoculated with small blocks of agar cut from the edge of a rapidly-growing culture of *Pythium ultimum*. Four plates each were placed at constant temperatures ranging from 4° to 36° C. at 4° intervals. After 24 hours diameters of the colonies were measured at right angles. This experiment was repeated 3 times, and the average diameter of the colonies in the 12 plates at each temperature are shown in figure 6. The average disease index after inoculation and percentage reduction in stand are presented for comparison. Best growth of the fungus in culture was at 28° with a slight reduction at 32° and 24°. The stand-reduction curve parallels the growth of the fungus in culture. The disease index differs in that there is a sharper rise from 16° to 20°, less from 20° to 24°, and no differences between 24° and 28°. However, the trend is the same with greater pathogenic activity of the fungus at temperatures near the optimum for its growth.
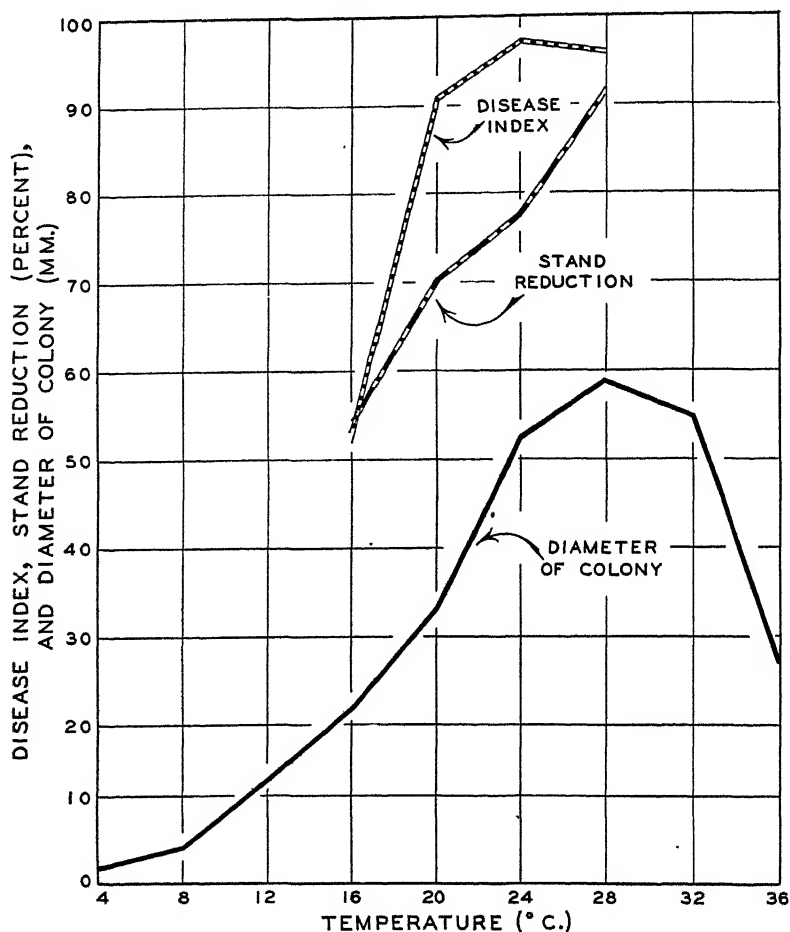
FIGURE 6.—Effect of temperature on diameter of colony of *Pythium ultimum* on potato-dextrose agar after 24 hours, on reduction of stand of peas in inoculated soil, and on disease index of bud-inoculated plants.

DISCUSSION

The effects of temperature on the foliage disease and on the damping-off phase in the range of temperatures tried indicate that increased severity at 24° to 28° is due principally to the effect of temperature on the fungus. There was a significant effect at the 5-percent level of preinoculation temperatures of the plants. The differences were small and the work would have to be repeated several times for the results to be accepted as conclusive.

In the field, the disease has been seen only after prolonged periods of cloudy and wet weather with at least one heavy rain in the period. Plants infected have ranged in maturity from those just emerged to those just beginning to blossom, but none have been seen infected after the blossom stage. This agrees with conditions for infection as

determined in the greenhouse. At average temperatures that would be expected during the early part of the season (16° to 20° C.) at least 48 hours of high humidity would be necessary for infection.

There is the question of how the inoculum gains access to the buds and leaf axils of the plant in the field. The fungus is common in the soil and has been reported frequently as an agent in damping-off of peas (*1, 10*). No fruiting bodies of any kind have been found on external parts of lesions; thus spread from plant to plant probably is negligible. Possibly sporangia or oospores are splashed from the soil surface and those lodging in water drops on the plants originate the disease if suitable moisture conditions prevail long enough. Since pea foliage is waxy, collection of water drops would be expected more frequently in buds or leaf axils. McLaughlin (*6*) isolated *Pythium* (species undetermined) from the soil surface in all seasons of the year with a higher percentage of total isolations belonging to *Pythium* in cooler months. The lowest percentage was under conditions of high temperature and low moisture.

Damage to the crop consists in delaying the maturity of affected plants beyond that of the surrounding ones. The entire plant is rarely killed. The pods produced on delayed branches are usually too late to be of any value when the crop is harvested for canning. Although local damage has been severe in some cases, widespread losses have not occurred since the disease has been under observation. Because of the conditions necessary for disease development and the fact that the fungus has undoubtedly been present for a long time without causing great damage, it is not expected that the disease will become a serious factor in production of the canning crop.

In the nursery in 1946 there were present a large number of breeding strains and varieties representing a wide range of types of canning peas. When the disease appeared the opportunity was taken to examine the various lots in detail for evidences of varietal differences in susceptibility. Infection was not entirely uniform, but the only differences that could be determined were that the disease was more prevalent in the lower and wetter parts of the nursery and on the shorter plants, regardless of variety. A second planting of one strain, just past emergence at the time of infection, had a much higher percentage of infected plants than the older, and taller, planting.

In the present investigation another species of *Pythium* has been shown capable of causing the same type of disease in the greenhouse as that caused by *P. ultimum* but with less severity. Probably other species could be found that would likewise be infectious under similar conditions. *P. ultimum* is the only species that has been isolated from diseased material. According to Middleton (*8*), *P. ultimum* is the commonest species of *Pythium*, and this may account in part for the fact that it was the only species isolated.

SUMMARY

A new disease of garden pea incited by *Pythium ultimum* is described. Infection occurs in the buds or in the axils of young leaves. Affected tissues collapse quickly and become straw-colored to black. Usually the upper three to five internodes are killed and new branches are put out below.

Typical disease was produced in the greenhouse by inoculating potted pea plants.

High humidity was necessary for infection.

Another species of *Pythium* was found able to cause the same type of disease but with less severity.

Temperatures from 24° to 28° C. were most favorable for disease development. The optimum for damping-off of pea seedlings and growth in culture was 28° C.

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THE EFFECT OF EARTHWORMS ON THE PRODUCTIVITY OF AGRICULTURAL SOIL[1]

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INTRODUCTION

Recent studies by the writers have brought out two points that bear on the relation of earthworms to agricultural soils: (1) Over widespread areas, the number of earthworms in crop fields is sufficient to influence markedly the structure of the soil (*10, 26*),[2] and (2) the harmful effect of row cropping on the earthworm population can be overcome by establishing a protective cover on the ground by late fall (*14*). These findings lay a partial ground work for the development of practices that maintain earthworms in row-cropped land. However, the advantage to crops that may accrue from the presence of earthworms has not been adequately investigated.

· It is recognized that earthworms have various chemical and physical effects on soil that should benefit productivity. They perforate the ground, thereby increasing aeration and infiltration (*3, 8, 9, 17, 18, 20, 24, 28, 29*). Their casts are relatively water stable, which should help in maintaining the structure intact under the dispersing action of rain water (*5, 7, 10, 12*). They are effective chemically in that they mix organic surface debris with the inorganic fraction to form humus material, thereby concentrating the nutrients in parts of the soil where they are more available for crop growth (*2, 3, 4, 9, 17, 18, 19, 21, 22, 23, 24, 25, 28, 29*).

Such observations of the effect of earthworms on soil have stimulated efforts to test the effect of earthworms on the yield of crops. Some of the first work along this line was reported in 1890 by Wollny (*30*), who found that when earthworms were added to soil in pots, marked increases in yield were obtained with a number of crops. Later workers (*1, 6, 17, 21, 24*) have repeated these experiments in one form or another. In general, the published results have confirmed Wollny's findings. When earthworms have been added to soil, either in pots or in the field, increases in yield have resulted.

Despite these favorable reports, agriculturists have hesitated to accept the results. This situation stems from at least four important and highly valid criticisms:

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[2] Numbers in parentheses refer to Literature Cited, p. 338.

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1. It has been reported in certain of the experiments that earthworms inoculated into the soil were found to have died by the end of the study. Since earthworms contain a high concentration of organic nitrogen, it has appeared that the beneficial effect of the inoculation on yield might be attributable simply to nitrogen fertilization. If the only effect is chemical, the same result could be obtained with chemical fertilizers and the earthworms would have no particular merit.

2. In others of the experiments, earthworm casts were collected and compared with the remaining soil in respect to the effect on crop yields. The criticism of this technique is that the casts contained much more of the organic matter and were therefore richer in the nutrients derived from plant residues. Agriculturists point to the well-known beneficial effect of incorporating organic matter into soil through cultural operations as evidence that the earthworms do nothing that cannot be done without them.

3. Other reports concern experiments in which soil was taken from areas that differed greatly in earthworm population and the productivity of these areas was compared under controlled conditions. The differences found in productivity have been ascribed by these investigators to the earthworms. Such experiments are justifiably subject to severe criticism in that they fail to demonstrate that the earthworms caused the good soil conditions. Agriculturists have been inclined to take the more straightforward viewpoint that the larger earthworm populations on better soils are due to more favorable conditions for their survival and reproduction.

4. The severe criticisms listed above are given added weight when it is considered that not all investigators have been able to obtain positive responses from inoculating earthworms under experimental conditions. It is true that the scientific reports are practically unanimous in showing positive effects. But negative results have been obtained, to the authors' knowledge. They are not reported in the literature probably because the investigations were abandoned at a preliminary stage.

The investigations reported in this paper were undertaken with a recognition of the criticisms to which previous work had been subject. Several tests were made under different conditions and variables were introduced that might help in clarifying the underlying causes of the responses shown by the crops. Owing to the diverse nature of the experiments, each will be described separately but they will be discussed and interpreted together at the end of the paper.

EXPERIMENTAL DATA

There are five experiments reported in this paper. In experiments 1 to 4 the species of earthworms used were a mixture of *Helodrilus caliginosus* forma *trapezoides* Duges and *Diplocardia* sp. Garman. In experiment 5 these species were used and in additoin *Lumbricus terrestris* L. and *Helodrilus chloroticus* Savigny. These are species commonly found in the vicinity of Beltsville, Md. The earthworms were stored for several days in the same soils as those used in the experiments in order to remove the previous contents of their alimentary tracts.

1. MILLET

Twelve glazed crocks were filled with a Christiana loam from which all earthworms had been removed. The soil was in good physical condition and had a moderately good organic content. Four sets were used as follows, with 3 replicates in each set:

Set 1. No worms added.
Set 2. 23 living worms placed in each of the crocks on May 10, 1946; these worms were removed on June 10, 1946.
Set 3. 23 dead worms placed in each of the crocks on May 10, 1946.
Set 4. 23 living worms placed in each of the crocks on May 10, 1946.

The worms were removed from the crocks of set 2 by spreading the soil on paper and examining it thoroughly. At the same time, all the other crocks were similarly handled. This treatment was somewhat similar to cultivation, since it left the soil in a crumbled condition.

On June 11, 1946, German millet was planted in the crocks at a rate equivalent to 50 pounds per acre. On July 8, the plants were thinned to 15 per crock. The millet was harvested on August 26, 1946.

During the period of the experiment, the crocks were placed in light shade outdoors and were watered occasionally.

The yield of millet in terms of oven-dry weight per stalk and the nitrate content of the soil on August 9, at which time the millet had ceased growth and was beginning to cure, is given in table 1. The

TABLE 1.—*Nitrate content of the soil and Millet yields at time of maturity for each crock*

Earthworm treatment	Oven-dry weight per millet stalk		Soil nitrate	
	Grams		*P.p.m.*	
1. None	2.3		4.1	
	2.1		3.8	
	1.8		3.2	
		2.1		3.7
2. Living earthworms for 30 days prior to planting millet	1.8		4.5	
	2.3		3.5	
	2.2		6.4	
		2.1		4.8
3. Dead earthworms	3.2		10.0	
	2.2		7.5	
	2.8		8.6	
		2.7		8.7
4. Living earthworms	3.7		7.5	
	2.6		7.5	
	2.8		5.6	
		3.0		6.9

number of stalks per crock varied slightly but without any relation to the treatments or to the weight per stalk.

The millet in the crocks that contained living worms throughout the period of the experiment had the highest average weight per stalk, 3.0 gm. However, the crocks with dead worms gave almost as high a value, 2.7 gm. The difference between the two sets was not significant. The crocks without worms or those from which the worms had been removed before the millet was planted showed much poorer growth, the average being 2.1 gm.

These differences in growth appeared to be associated in part with the nitrate content of the soil. The highest nitrate concentration was

in the crocks receiving dead worms, 8.7 p.p.m. In the crocks with living worms, the concentration was somewhat less, 6.9 p.p.m. Still, this value was almost twice that for the uninoculated crocks. The crocks from which the living worms had been removed prior to planting showed only slightly more nitrate than the check.

The high nitrate concentration in the crocks receiving dead worms had an obvious explanation; the nitrate was released in the decomposition of the worms' bodies. But the high nitrate concentration in the crocks containing live worms was not explainable until the soil was removed and examined for earthworms. It was found to contain an average of nine eggs and young worms per crock. Evidently the mature worms placed in the crocks had reproduced and died. Since such a reproductive phase is a normal occurrence in the life cycle of earthworms in the field, the phenomenon observed in the crocks had practical significance. This application will be considered in the discussion.

2. LIMA BEANS

The same 12 crocks were used for this test as for test No. 1. After the millet was harvested and the earthworms counted on August 26, 1946, the soil was put back in the crocks. The crocks were then placed outdoors in an unsheltered location. They were left fully exposed to the elements throughout the winter of 1946–47, and the following summer. On December 10, 1947, they were brought into the greenhouse and cultivated to a depth of about one-half inch. There was no evidence of living earthworms in the crocks. Calcium carbonate equivalent to 1 ton of limestone per acre was worked into the cultivated soil.

The 12 crocks were rearranged into 3 sets. The 4 crocks in each set consisted of 1 from each of the 4 variables of the millet experiment. In this way, any possible hold-over effect of the millet experiment on the growth of lima beans could be detected. However, no such effect was noted and this point will therefore be disregarded in the ensuing discussion. Apparently, the one and a half years the soil was exposed without earthworms to the elements served to overcome any differences that may have remained at the end of the millet experiment.

The three sets were treated as follows:

Set 1. No worms added.
Set 2. Ten dead earthworms placed in each of the crocks on December 10, 1947.
Set 3. Ten living earthworms placed in each of the crocks on December 10, 1947.

The greenhouse was maintained at natural temperature with artificial heating only when necessary to prevent freezing. The crocks were watered as necessary during the period of the experiment. There was considerable evidence of earthworm activity in the inoculated soil during the winter period. On April 22, 1948, the soil was fertilized with 4–8–12 at the rate of 1,000 pounds per acre, and one dwarf lima bean planted in each crock. The beans were harvested on August 2, 1948. The yields are shown in table 2.

The highest average yield was obtained in the set that had been inoculated with living earthworms. The set containing dead earthworms yielded almost but not quite as much. As in the millet ex-

TABLE 2.—*Yields of lima beans and pods in each crock*

Earthworm treatment	Air-dry weight of pods		Air-dry weight of beans	
	Grams		*Grams*	
1. None	7.3		5.0	
	6.8		4.7	
	0.8		0.0	
	8.5		5.9	
		5 8		3.9
2. Dead earthworms	23.4		14.8	
	16.3		11.3	
	5 6		3.3	
	19 0		13.0	
		16.0		10.6
3. Living earthworms	11.0		7.0	
	15.5		10.3	
	14.7		9.6	
	26.3		19.2	
		16.9		11.5

periment, the difference between these two sets was not significant. But the set without earthworms showed very much poorer growth. These differences are shown in figure 1. This experiment indicated

FIGURE 1.—Lima bean experiment: *A*, No earthworms added; *B*, dead earthworms added; *C*, living earthworms added. This soil was in good structure and showed a markedly stimulating effect from the chemicals present in the bodies of the earthworms but only a slight additional effect from the activity of the living earthworms.

that the chief effect of the earthworms could be attributed to chemicals in their bodies. But there was a suggestion of a slight additional effect from the action of living earthworms on the soil.

3. SOYBEANS

Ten wooden frames, measuring 8 inches long, 3 inches wide, and 6 inches high, and fitted with a glass front, were filled with the same type of soil as that used in the millet and lima-bean experiments. All worms were removed from the soil before it was placed in the frames. The soil was treated with 4–8–12 at a rate corresponding to 1,000 pounds per acre and hydrated lime at 2,000 pounds per acre. The test was started on June 3, 1947, at which time earthworm inoculations were made. The frames were stored in a cold-storage room at 55° F. until September 9, when they were placed under light shade in a greenhouse. One Black Wilson soybean was planted in each frame. The vegetation was harvested on November 26, 1947. Five sets were used with each set in duplicate, as follows:

Set 1. Soil maintained in its natural structure; no worms added.
Set 2. Soil puddled to destroy its natural structure: no worms added.
Set 3. Soil puddled, 10 dead worms added to each frame on September 9, 1947, the day the soybeans were planted.
Set 4. Soil puddled, 10 living worms added to each frame on June 13, 1947, approximately 3 months before the soybeans were planted.
Set 5. Soil puddled, dried, and broken into crumbs to simulate restoration of its original structure.

The oven-dry weight of above-ground vegetation is given in table 3. The soybeans grew poorly in the puddled soil and in the puddled soil in which dead earthworms were placed. They grew much better where the natural soil structure had been maintained or had been restored artificially, as well as where living earthworms were placed in the soil. The results indicate that the benefit from living earthworms on the soybeans grown in this soil was associated with the physical action of the earthworms on the structure of the puddled soil rather than the release of nutrients from their bodies.

Table 3.—*Soybean yields in each frame*

Soil and earthworm treatment	Oven-dry weight of tops
	Grams
1. Natural soil structure	{ 4.20 1.71
	—— 2.96
2. Soil puddled	{ .56
	--
	—— .56
3. Soil puddled, dead earthworms added when soybeans were planted	{ .52 .79
	—— .66
4. Soil puddled, living earthworms added	{ 2.48 2.13
	—— 2.30
5. Soil puddled, structure artificially restored	{ 2.30 3.82
	—— 3.06

4. WHEAT

The same 10 frames were used for this test as for test No. 3. After the soybeans were harvested on November 26, 1947, the frames were left for several days. Then, on December 5, they were fertilized with 4–8–12 at the rate of 500 pounds per acre and fresh cow manure at the rate of 10 tons per acre. These materials were worked into the top one-half inch of soil. The frames of set 4 were reinoculated with 10 living worms each. On December 15, 10 dead worms were worked into the surface soil in the frames of set 3. The next day, 3 wheat grains were planted in each frame. During the winter, the plants were kept in a greenhouse at natural temperature with artificial heating only when necessary to prevent freezing. The wheat grew slowly during the winter, but in the spring became active again. Differences in growth began to appear among the sets and it became evident that the better growth was taking place in the frames that had been inoculated with living earthworms. In order to preclude the effects of the ordinary nutrients, all frames were refertilized on March 11, 1948, with 10–6–4 in liquid form at the rate of 300 pounds per acre, and on March 19, with fresh manure at the rate of 10 tons per acre. The wheat matured normally and was harvested on June 16, 1948. The yields are given in table 4. The number of wheat grains parallel the weight of the stalks fairly well.

The puddled soil (set 2) showed distinctly poorer growth despite the heavy fertilization and manuring. The addition of dead earthworms increased the yield, but the highest average yield was obtained with the living earthworms. These data indicate that the wheat benefited from chemicals in the earthworms' bodies other than those in the fertilizer and the fresh manure that had been applied, as well as from the effect of the living earthworms on the soil.

TABLE 4.—*Wheat yields in each frame*

Soil and earthworm treatment	Air-dry weight of stalks		Wheat grains	
	Grams		*Number*	
1. Natural soil structure	9.4 9.7	9.5	109 132	120
2. Soil puddled	8.7 5.5	7.1	80 41	60
3. Soil puddled, dead earthworms added when wheat was planted	8.1 10.0	9.0	82 103	92
4. Soil puddled, living earthworms added	9.4 11.5	10.5	131 88	110
5. Soil puddled, structure artificially restored	7.7 10.9	9.3	94 80	87

5. MIXED HAY

The experiment with mixed hay was conducted on an earthworm-free clay subsoil in barrels that measured 32½ inches in height and 20 inches top diameter (fig. 2). The barrels were placed outdoors

FIGURE 2.—Hay experiment: *A*, Dead earthworms added; *B*, living earthworms added. Suitable cover conditions were maintained in both barrels for the winter survival of earthworms. The soil was in poor structure and showed a large stimulating effect on clover from the addition of living earthworms.

throughout the period of the experiment so that their climatic environment was fairly natural. The data reported in this paper are only a part of the experiment, the remainder is not pertinent. The procedure will be described only briefly since details have been given in a previous article (*16*).

In the fall of 1946, the soil in the barrels was liberally limed, manured, fertilized, and seeded to a mixture of grasses and legumes. Despite the treatments, the barrels supported only a moderate growth of weedy vegetation in 1947 because of the original unproductivity of the soil. In the fall of 1947, living earthworms were placed in the soil of 10 out of the 20 barrels that are described in this article. The introduction was equivalent to a population of 339,000 per acre. The other 10 barrels, which served as checks, were inoculated with the same number of dead earthworms. In April 1948, after the soil had thawed, the barrels were again inoculated with earthworms.

The barrels were treated by pairs, one of each pair having received living earthworms, the other not. The treatments applied to the pairs were as follows:

Set 1. Sod clipped in the fall of 1947, clippings left on surface.
Set 2. Same as set 1, except in addition, top-dressed with 300 pounds per acre of 4–8–12 in the fall of 1947 and again in the spring of 1948.

Set 3. Sod clipped in the fall of 1947, clippings removed, and soil surface either covered with asphalt roofing paper or left bare.

Set 4. Same as set 3, except in addition, top-dressed with 300 pounds per acre of 4–8–12 in the fall of 1947 and again in the spring of 1948.

The hay was harvested from the barrels in June of 1948. Yields are shown in table 5.

In sets 1 and 2, where the clippings were left on the surface in the fall so as to keep the earthworms alive over the winter (*11, 14*), the barrels inoculated with living earthworms bore almost four times as much vegetation as the barrels inoculated with dead earthworms (fig. 2). The effect of the living earthworms was somewhat greater in the barrels receiving a top dressing of fertilizer in the spring. In sets 3 and 4, where the soil surface was left either bare or with only a light asphalt paper covering over winter, inoculation with living earthworms had much less effect on yield.

TABLE 5.—*Hay yields in each barrel*

Overwinter cover	Top-dressed in spring	Air-dry weight of vegetation		
		Inoculated with living earthworms	Not inoculated with living earthworms	Apparent effect of inoculation
		Grams	*Grams*	*Grams*
Sod clippings	No	64.3 67.0 —— 65.6 103.7	13.1 16.1 —— 14.6 27.8	51.0
Do.	Yes	74.7 —— 89.2	26.9 —— 27.4	61.8
None or asphalt paper	No	25.0 26.2 58.3 —— 36.5	7.6 9.8 15.6 —— 11.0	25.5
Do.	Yes	21.5 28.8 60.8 —— 37.0	19.0 23.2 [1] 55.0 —— 32.4	4.6

[1] This barrel infested with ants.

The beneficial effect of the living earthworms in the barrels with suitable cover conditions seemed to be due to their physical action on the structure of the soil. An ant infestation, which occurred in the early part of 1948 in one of the barrels, had a similar stimulating effect. It appears, however, that under outdoor conditions this physical action of earthworms is effective primarily during the winter season; for the reinoculation, made in the spring of 1948, did not overcome the loss of the earthworms in the barrels that had insufficient late fall protection.

The stimulation of the vegetation under the action of the earthworms was confined almost entirely to the growth of clover. Where living earthworms were not introduced or where they were added but unsuitable cover conditions established for their survival, the vegetation was chiefly grass, weeds, and later in the summer, lespedeza. Where the earthworm population was favored, the growth was largely clover with only a small proportion of grass, weeds, and lespedeza. The infestation by ants likewise stimulated the clover.

DISCUSSION

The results of these various experiments are brought together in table 6. Since the same set of variables was not used in every experiment, only the comparisons between no earthworms, dead earthworms, and living earthworms are given in the summary table.

The addition of dead earthworms increased the yields by an average of 81 percent. The trend was consistent for all the experiments, although the actual magnitude of the increase was small in the soybean experiment.

TABLE 6.—*Summary of the experiments*

Experiment	Yields of vegetation			Benefit of—	
	No earth-worms	Dead earth-worms	Living earth-worms	Dead earth-worms over no earth-worms	Living earth-worms over dead earth-worms
				Percent	*Percent*
1. Millet: Weight per stalk_____grams__	2. 1	2. 7	3 0	29	11
2. Lima beans:					
Beans_____grams__	3. 9	10. 6	11. 5	175	8
Pods_____grams__	5. 8	16. 0	16. 9	183	6
3. Soybeans: Weight of tops_____grams__	. 56	. 66	2. 30	18	248
4. Wheat:					
Weight of stalks_____grams__	7. 1	9 0	10. 5	27	17
Number of grains_____ _____	60	92	110	53	20
5. Hay (sod clippings over winter): Weight of vege-					
tation_____ _____grams__	_____	21. 0	77. 4	_____	269
Mean_____	_____	_____	_____	[1] 81	[2] 83

[1] Average increase in yield due to dead earthworms.
[2] Additional increase in yield due to living earthworms over the increase due to dead earthworms.

However, the chemicals added in the bodies of the earthworms were not the entire explanation of the effects of earthworms on yield. Although the dead earthworms consistently increased yields over no earthworms, the living earthworms had a marked additional effect. The yield with living earthworms was 83 percent greater than with dead earthworms. This effect was consistently positive in all experiments. It was especially marked in the soybean and hay experiments, and rather slight in the other experiments.

The variation in the effect of living earthworms as compared with dead earthworms was explainable by crop and soil differences. If, for the moment, we disregard the hay experiment, we may consider the other four experiments as a unit, for all were conducted on soil from a single field. Originally, this soil had good structure and contained a large earthworm population. In the millet and lima-bean experiment, the soil was placed in the containers without destroying its aggregate structure. In the soybean and wheat experiments, the soil was puddled in order to destroy its structure.

The soybeans and wheat responded differently to the loss of structure. Puddling reduced the yield of soybeans 81 percent (from 2.96 to 0.56 gm., as shown in table 3). But the yield of wheat was reduced by puddling only 25 percent (from 9.5 gm. to 7.1 gm., as shown in table 4). The addition of dead earthworms to the puddled soil bene-

fited the soybeans only slightly, but the wheat markedly. Evidently soil structure was limiting the growth of soybeans to such an extent that they could not respond to the chemicals provided in the earthworms' bodies. With the wheat, where structure was having only a slightly depressive effect, the plants were able to respond to the addition of the chemicals.

The difference among crops in their tolerance to poor soil structure, as exemplified herein by soybeans and wheat, is evidently a factor that must be considered in evaluating the effect of earthworms on yield.

The millet and lima beans, growing in soil of good structure, responded markedly to the addition of dead earthworms. Here the structure was not limiting and the chemicals added through the bodies of the earthworms had a stimulating effect on growth. The living earthworms increased the yield only slightly over the dead earthworms, and the difference in favor of living earthworms was not significant with either crop. Yet it was consistent in both and therefore probably real. There was evidently a small additional benefit from the activity of living worms.

The hay experiment differed from the four others as regards the soil used. It was an extremely unproductive clay subsoil of very poor structure. The growth of hay was poor despite the cultivation given this soil in the original preparation of the seedbed; the very liberal use of lime, fertilizer, and fresh manure; and the addition of dead earthworms. The factor retarding growth was evidently not the lack of chemicals. But when living earthworms were introduced with favorable cover for their survival, vegetation was greatly stimulated. The beneficial effect was associated with improved structure as indicated by infiltration tests as well as by a similar effect from the infestation of ants that likewise loosened the soil. The response was mainly in the growth of clover rather than in the grass, weeds, or lespedeza. This experiment, then, gave further evidence that the difference among crops in their tolerance to poor soil structure is a factor in the effect of earthworms on yield.

Since both living and dead earthworms had stimulating effects according to the limiting factors of the soil, consideration should be given to the manner in which these effects may operate in the field.

In a previous study (*11*) it has been shown that earthworms follow a life-cycle pattern related to ecological factors of the soil. In order to clarify these relationships, one of the illustrations of the earlier study is reproduced herein (fig. 3). As shown in the illustration, earthworms reach maturity during the winter and spring months and then reproduce. The parent generation largely dies during the summer and is replaced by a new generation. On cultivated land that has insufficient protection in the late fall to maintain the earthworms, they die in early winter.

The present study suggests that the maintenance of the normal life cycle, characterized by death of the mature earthworms during the summer, may be important to the growth of crops. The beneficial chemicals in the dead earthworms would then be released at a time when crops are able to use them. On the other hand, the upset in this normal cycle, when late-fall protection is insufficient, results in
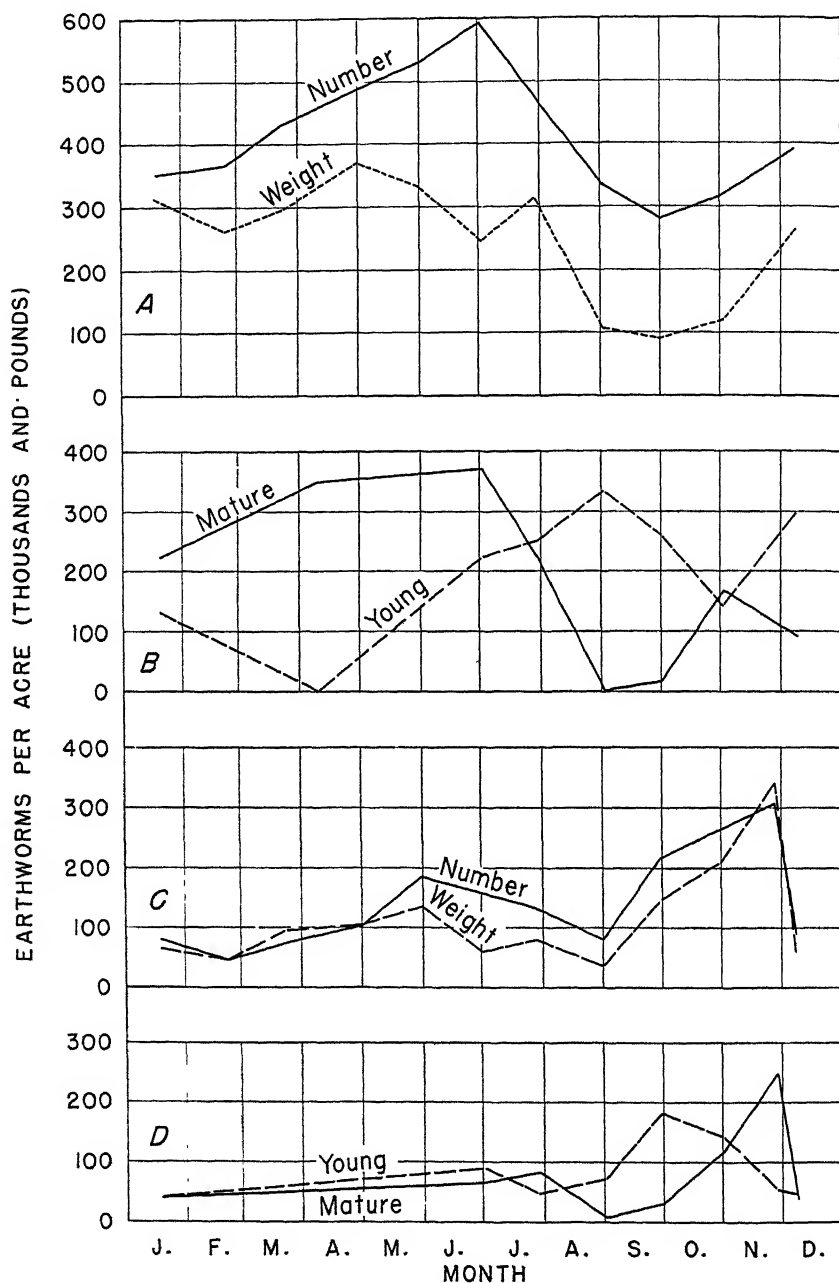
FIGURE 3.—The annual earthworm cycle in a field at College Park, Md.   On sod ground (*A* and *B*), the mature earthworms reproduced and died during the summer; on cultivated ground that had insufficient protection in the late fall (*C* and *D*), the earthworms for the most part died in the early part of the winter.

the release of these chemicals at a time when crops are dormant and unable to use them.

Identification of the stimulating chemicals requires further investigation. As shown in the millet experiment, the death of the earthworms released a noticeable amount of nitrate; and nitrate was probably the most critical nutrient in this soil. The release of nitrate and other ordinary essential elements from the earthworms' bodies may account in part for their beneficial chemical effect. But in all the experiments, liberal quantities of commercial fertilizer were used and yet the dead earthworms increased yield. In the wheat experiment, fertilizer was applied in the fall and again in the spring, the total application being equivalent to 50 pounds of N, 58 pounds of P_2O_5, and 72 pounds of K_2O per acre. In addition, 10 tons per acre of fresh cow manure was worked into the soil in the fall, and 10 tons more applied as top dressing in the spring. Still the dead earthworms increased yield. It therefore appeared that the beneficial chemicals in the bodies of earthworms, whatever their nature, produced effects that were not obtained from the commercial fertilizer or the fresh cow manure used.

Also the beneficial physical activity of living earthworms is dependent on the maintenance of their normal life cycle. In the present study, it was found that the activity of living earthworms was especially effective in soil with poor structure. The poor structure was attained in experiments 3 and 4 by artificially puddling a well-aggregated soil, and in experiment 5 by selecting a clay subsoil that was of extremely poor structure. In this respect, therefore, the soils were not typical of most soils used for agriculture. However, it is widely recognized that the loss of soil structure often does become a limiting factor under field conditions. In two papers (*16*, *27*) published in conjunction with this paper, it is shown that much loss of structure is associated with the winter season and that the maintenance of the earthworm population during the winter is associated with benefits to soil structure. This physical activity was found in the hay experiment of the present paper (experiment 5) to be effective with earthworms introduced in the fall and not with those introduced in the spring. While earthworms can affect soil structure at almost any time of the year, if suitable moisture and temperature conditions are provided, the evidence from these experiments and field observation indicates that under actual farming conditions their physical activity in soil occurs largely from fall to spring. During the summer, when the soil becomes warm and dry, earthworms have much less influence on the structure of the soil. The benefits of earthworms on soil structure are therefore dependent on maintaining the population over winter.

SUMMARY

Tests were made to determine the effect of earthworms on the productivity of soil.

In five tests, with different crops and soils, earthworms consistently increased yields. Their influences varied widely according to crop and soil.

The increases in yield were attributable to the release of beneficial

chemicals from the bodies of the earthworms and to their physical effects on soil structure.

The beneficial chemicals were not identified, but their effects were not offset by liberal application of N–P–K fertilizer and fresh cow manure.

Available evidence suggests that the release of beneficial chemicals occurs principally in the summer season when earthworms normally pass through their reproductive period.

Beneficial physical effects on soil structure were obtained on soils where the lack of structure was limiting to crop growth.

The crops differed in their tolerance to poor soil structure and therefore in their response to the activity of living earthworms. Soybeans and clover were stimulated more than wheat and grass by the activity of living earthworms in soil of poor structure.

Benefits from the physical activity of living earthworms were obtained at various seasons depending on favorable soil moisture and temperature, but available evidence indicates that under the field conditions examined, this activity is largely effective during the late fall, winter, and early spring.

In one of the experiments, with soil of poor structure, ants produced beneficial effects on yield similar to those of earthworms; an observation which suggests that soil fauna other than earthworms may also have far-reaching importance to the productivity of soils.

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THE ACTION OF FROST ON THE WATER-STABILITY OF SOILS [1]

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INTRODUCTION

Soil that had some form of insulative protection throughout the winter months was found recently to be in better physical condition the following spring than soil that lay bare over winter (*9*).[2] Infiltration rates and water-stability were higher for the protected soil. These benefits were associated in part with increased earthworm activity (*4, 6*). On the unprotected soil, there was evidence that the structure had deteriorated during the winter months. The deterioration appeared to be related to the action of frost on the unprotected soil.

Few studies have been made of the effect of frost on soil. Baver (*1*) states that the effect of freezing and thawing is to granulate clods in the soil but adds that it does not always result in a highly aggregate soil. He cites investigations by Jung (*7*) in which soil moisture content and rate of freezing were found to modify the action of frost. The data appear to involve the action of frost on the flocculation of soil colloids. However, it is not clear that the data have any relation to the formation of soil aggregates. Likewise the studies by Gardner (*5*) concern the action of frost in flocculating dispersed colloids.

These previous works show that freezing and thawing affect soil, but whether the effects are beneficial or detrimental to aggregate structure is not clear. In view of the field observations made by the writers and the uncertainty in the literature, it was considered desirable to make a specific study of the effect of frost on soil structure.

EXPERIMENTAL PROCEDURE

The soils used for the study were obtained from experimental plots at Wooster and Holgate, Ohio; and at College Park, Md. The soil from Holgate was a clay loam of lacustrine origin, that from Wooster was a glacially derived noncalcareous silt loam. The soil from College Park was also a silt loam, but derived from coastal plains deposits. At each location two samples of soil were selected. They were of the

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[2] Numbers in parentheses refer to Literature Cited, p. 346.

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same soil type, but differed in physical quality as shown by water-stability tests. The differences in quality were the result of previous cropping practices.

Samples of dry soil weighing 100 gm. were placed in covered metal cans. Water was added to the samples slowly in measured amounts. Three different moisture levels were tested. The lowest level was equal to the moisture equivalent. The others were equal to 15 and 30 percent of moisture in excess of the moisture equivalent. After the water had distributed itself uniformly there appeared to be no free water in any of the samples.

The samples are divided into six treatment groups as follows:

1. Frozen and thawed rapidly one time.
2. Frozen and thawed slowly one time.
3. Frozen and thawed rapidly five times.
4. Frozen and thawed slowly five times.
5. Moistened and dried immediately.
6. Stored moist at 50° F. for the duration of the freezing tests.

Freezing was accomplished by placing the cans in a cold storage room at a temperature of 0° F. The slow rate of freezing was obtained by wrapping the cans in cotton batting and placing them in cardboard containers. The soil froze in about 5 hours. Samples for fast freezing were not insulated. They froze in about 1 hour.

Freezing caused an expulsion of considerable free water from the wettest samples. When the freezing tests were complete, the excess of free water was pipetted off and measured.

After this treatment, the frozen and unfrozen samples were allowed to air-dry at room temperatures and were crushed to pass a 5-mm. screen. The 5-3 mm. fractions were analyzed to determine water-stability (*3*). Water-stability is used in this study to show the effects of freezing on soil structure.

RESULTS

The soils frozen at high and at medium moisture content showed obvious evidence of physical damage. They were in a puddled condition and markedly slumped. Free water had been expelled by freezing (*2*). In the wettest samples it completely covered the surface of soil. In 26 out of the 48 samples, frozen at high moisture content, the free water amounted to more than 10 percent of the total that had been added.

The puddled condition of the samples as a result of freezing is reflected in the water-stability data which are given in table 1. The data were analyzed statistically, and effects of importance are presented in smaller summary tables.

TABLE 1.—*The water-stability of soils as affected by treatments*

Treatment	Soil from—					
	Holgate		Wooster		College Park	
	Poor quality	Good quality	Poor quality	Good quality	Poor quality	Good quality
Low moisture	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Dried immediately	48.0, 41.8	76.7, 76 8	31.3, 26 7	65 5, 68 0	39 3, 34.4	49 5, 56.2
Stored moist	54.0, 51.2	78.0, 74.1	29 9, 31 5	75 2, 69 6	35.3, 38 1	60.4, 63.5
Frozen rapidly, once	42 4, 46.3	73 8, 74.0	13.9, 20.1	58.1, 65.8	30 2, 34.6	58 0, 64.1
Frozen rapidly, 5 times	36.1, 37.6	63 2, 62.0	16.0, 19.9	61.0, 59 8	32 7, 30.4	55 8, 58 9
Frozen slowly, once	44 0, 43 2	73.6, 76.5	19.9, 21 8	59.1, 65.0	29.8, 34 5	58 2, 51.8
Frozen slowly, 5 times	41 8, 36 0	75 0, 71.8	17 7, 19.1	57 7, 66 5	33.7, 32 2	60 8, 58.2
Medium moisture						
Dried immediately	38.9, 34 8	70 8, 71.2	25 3, 19.1	61.6, 59 3	25 5, 24 5	53.7, 46.1
Stored moist	38 4, 43 4	71.5, 74.0	24.5, 23 1	57.3, 64.1	28.8, 29.4	56.5, 56.6
Frozen rapidly, once	27.9, 28 4	59.2, 58.5	10 3, 11.7	55.0, 54 3	20.8, 22.6	46.5, 42 1
Frozen rapidly, 5 times	16 1, 22 8	39.2, 34.1	10.9, 12 6	46.7, 48.7	16.4, 14.4	28 5, 34 9
Frozen slowly, once	27.1, 28.8	55 4, 55.1	14.4, 15 7	50.4, 61 8	17.2, 19 5	31 1, 34.8
Frozen slowly, 5 times	19.2, 22.6	41.5, 40.2	11.6, 14 5	40.7, 45 8	20 7, 16.6	29 8, 33.9
High moisture						
Dried immediately	30 9, 27.3	60 7, 64.2	17 6, 16.4	55.6, 61.1	19 7, 18.9	40 7, 44.0
Stored moist	29 5, 34.6	66 7, 69 9	16.5, 17.9	58.6, 63 3	22 4, 19 3	49.6, 43.2
Frozen rapidly, once	16 0, 16 6	39.0, 39.3	6 1, 8.5	32.8, 42 4	14 7, 13 2	25.6, 26 7
Frozen rapidly, 5 times	8.5, 9.7	23.5, 26.8	7.1, 8 0	28 5, 31 5	11.9, 9 2	15.5, 18.5
Frozen slowly, once	14.3, 17.5	46 1, 40.0	8 9, 8 2	39.1, 44 2	14 6, 13.9	18.6, 24.3
Frozen slowly, 5 times	10 5, 10.0	21.2, 26.3	6.9, 7.3	29.7, 23.8	10 8, 10 2	16.4, 20.1

Data on the effect of storing or freezing moist soil are summarized in table 2. The frozen soils suffered an average decrease in water-

TABLE 2 —*The effect of storing and freezing moist soils*

Soil treatment	Water-sta-bility	Effect of treatment
	Percent	*Percent*
Moistened and dried immediately	45	
Stored moist	48	[1] 3
Frozen moist	33	[1] —12

[1] Significant beyond the 1-percent level.

stability of 12 percent, for all conditions of freezing. This decrease was attributable directly to the freezing and not merely to the moistening of the soil, for the soil that was stored moist actually increased in water-stability by an average of 3 percent. The decrease due to freezing and the increase due to moist storage were both highly significant statistically.

The effect of freezing on different types and qualities of soil is shown in table 3. All soils showed significant reductions in water stability as

TABLE 3.—*The effect of freezing soils of different types and qualities*

Soil type	Soil quality	Water-stability		
		Dried im-mediately	Frozen	Effect of freezing [1]
		Percent	*Percent*	*Percent*
Molgate	Poor	37	26	—11
	Good	70	51	—19
Wooster	Poor	23	13	—10
	Good	62	49	—13
College Park	Poor	27	21	—6
	Good	48	38	—10

[1] All effects significant beyond the 1-percent level.

a result of freezing. Soils of good quality suffered a larger reduction in water-stability than soils of poor quality. The better quality soils had a higher water-stability to begin with, hence there was more material to be disintegrated by freezing. However, the water-stability after disintegration by freezing remained higher in the better soils.

The moisture content at which the soils were frozen had a marked effect on the reduction in water-stability. Results are summarized in

TABLE 4.—*The effect of freezing at different moisture contents*

Moisture content above moisture equivalent	Water-stability		
	Dried immediately	Frozen	Effect of freezing [1]
Percent	*Percent*	*Percent*	*Percent*
0	51	47	−4
15	44	31	−13
30	38	20	−18

[1] All effects significant beyond the 1-percent level.

table 4. Freezing at the moisture equivalent caused only a small decrease in water-stability, whereas the greatest loss in water-stability was incurred at the highest moisture content.

Rate of freezing and thawing (table 5) had no significant effect on water-stability under the conditions of this experiment.

TABLE 5.—*The effect of slow and fast rates of freezing*

Moisture content above moisture equivalent	Water-stability		
	Frozen slow	Frozen fast	Effect of fast freezing [1]
Percent	*Percent*	*Percent*	*Percent*
0	48	46	2
15	31	32	−1
30	20	20	0

[1] All effects are nonsignificant.

Freezing and thawing repeatedly (table 6) decreased water-stability

TABLE 6.—*The effect of number of times of freezing*

Moisture content above moisture equivalent	Water-stability		
	Frozen once	Frozen 5 times	Effect of repeated freezing
Percent	*Percent*	*Percent*	*Percent*
0	48	46	−2
15	35	28	[1]−7
30	24	16	[1]−8

[1] Significant beyond the 1-percent level.

to a greater extent than freezing and thawing once. The difference was not significant at the lowest moisture content, but was highly significant at the higher moisture contents.

The above data show that the most drastic treatment was to freeze the soil repeatedly at a high moisture content. Soils subjected to this treatment showed very little remaining water-stability; they were almost completely dispersed. The data show that some water stability is left, but actually the material remaining on the sieves was mostly discrete sand particles. These were not distinguished from aggregated particles in the method of analysis that was used (*3*).

DISCUSSION

The damaging effect of frost on the water-stability of soils undoubtedly occurs frequently under field conditions. The puddled appearance and slumped condition of frozen soil as observed in this investigation can be seen in many fields in early spring, particularly those that lay bare over winter. According to the results of this study, one would expect to find the greatest damage where soils are frozen and thawed repeatedly, and where their moisture content is high.

In this connection the work of Post and Dreibelbis (*8*) is of interest. They found that a concentration of water at or near the soil surface is a characteristic of frozen soils, and that upon thawing the moisture content at the surface tends to approach saturation. It follows that to prevent increasingly greater damage by repeated freezing such excess water must be drained from the soil surface.

This study on the factors that influence the effect of frost on soils, and the writers' previous investigations (*6, 9*), suggest a means of moderating frost damage. Insulative protection of mulch or vegetation reduces the number of times a soil freezes, and the depth to which frost penetrates. It promotes and maintains large populations of earthworms. These increase infiltration, thereby allowing the drainage of excess water out of the surface soil. By favoring the activity of earthworms the hazard of freezing at high moisture contents is reduced.

The uniformity with which frost damaged the water-stability in this study leads the writers to question whether frost ever promotes good structure in agricultural soils. It is true that the physical condition of protected soils often improves over winter. This may erroneously be attributed to frost action. Actually, improvement is due to other favorable factors, such as the protection from freezing and the stimulation of earthworm activity.

The writers in their experience have yet to find any structural improvement in fields that lay bare over winter. It has been noted that when clay soils are plowed in the fall, they are frequently friable and easily tilled in the spring. The frost action fractures the intractable lumps and clods. By the same token, bonds within the aggregates are likewise destroyed. The result is that the soil, while apparently in good condition because of its freedom from clods, actually has so little water-stability remaining that the favorable tilth produced by land preparation is not retained throughout the growing season. For this reason, attempts to maintain good structure in heavy soils by fall plowing have notably failed.

SUMMARY

Freezing and thawing decreased the water-stability of moist soils. The detrimental effect of freezing and thawing was most marked at high moisture contents.

The loss of water-stability was increased by repeated freezing and thawing.

Rate of freezing and thawing had no effect on the loss of water-stability.

Methods designed to overcome the harmful effects of frost action should be based on reducing the depth and frequency of freezing, and on maintaining drainage channels in the soil layer where freezing takes place in order to keep the moisture content as low as possible.

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A PRINCIPLE FOR MAINTAINING STRUCTURE IN CLEAN-CULTIVATED SOILS [1]

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INTRODUCTION

The deterioration in structure that occurs when soil is clean-cultivated presents a widespread problem in conserving the productivity of agricultural land. The problem becomes acute primarily in loam and clay soils (*9*),[2] but it occurs to a noticeable extent in some of the lighter soils as well (*3, 8*). Deteriorated structure is found in, but not confined to eroded soil. It is an inevitable accompaniment of clean cultivation. Numerous studies (*1*) have demonstrated that when soil is subjected to clean cultivation, it tends to become more compact. The volume of large pores decreases, thereby adversely affecting the intake of water and the normal growth of roots. Where such conditions obtain, drought and nutrient deficiencies become evident despite adequate natural precipitation and the liberal use of fertilizer (*4*).

The loss of structure that accompanies clean cultivation is usually associated with various other changes in the soil. The most important of these are a decrease in organic-matter content, aggregation, nitrogen, and exchangeable bases (*1*). However, the extent to which these changes are actually responsible for poor structure or are only parallel accompaniments has been difficult to ascertain.

With the lack of critical information on the direct causes of the loss in structure, progress in preventing this deterioration has been slow. Manure and lime have been demonstrated to have beneficial effects, but in general there is no practical method for preventing loss in structure when land is clean-cultivated. Therefore farmers located on soils susceptible to such loss have to rely principally on sod crops to restore structure after each period of clean cultivation. A result of such rotating is an increase in the total acreage required for the production of clean-cultivated crops and an increase in the economic pressure on land of lower capability and greater erodibility.

In examining clean-cultivated land for clues as to the direct cause of the loss of structure, it was observed that much of the damage appeared to occur during the winter season. A laboratory study was conducted on three soils from widely separated localities to determine

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[2] Italic numbers in parentheses refer to Literature Cited, p. 352.

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the effects of freezing and thawing on structure (*10*). It was found that this climatic process destroyed much of the aggregate structure when the soil was at a high moisture content. However, the damaging effect was much less at lower moisture contents. At the moisture equivalent (18 to 30 percent of moisture, depending upon the soil), freezing and thawing damaged the aggregate structure only slightly.

Solution of the problem, then, appeared to depend on the removal of excess water during the winter from the surface soil as deep as the frost line. It appeared that this might be accomplished by stimulating a natural development of drainage. The writers had found in other studies (*7*) that earthworm holes serve as drainage channels, and that the activity of earthworms during the winter improves the infiltration capacity of soil. However, on clean-cultivated land, they are usually killed during the winter by the lack of sufficient surface protection (*5, 6*).

The present study was undertaken to determine whether the structure of clean-cultivated soil would be maintained by protecting the ground over winter, thereby decreasing depth and frequency of freezing, and stimulating the activity of the earthworms in making holes to remove excess water during the critical freezing periods.

PROCEDURE

The study was conducted on field plots located at the Maryland Agricultural Experiment Station near College Park, Md., the Ohio Agricultural Experiment Station, Wooster, Ohio, and the Northwest Experimental Farm, Holgate, Ohio. The plots had been in operation by the respective cooperating agencies for a minimum of 7 years. At each location, plots in the following crops were selected:

1. Continuous corn, stover removed;
2. Continuous corn, organic matter returned as manure or stover;
3. Corn in rotation, stover removed;
4. Wheat, followed by clover or alfalfa in rotation;
5. Continuous sod hay.

There were some differences among the locations in details of the cropping and fertilizer methods. These differences may have influenced the result somewhat, but since the purpose of the study was to determine effects of general applicability, the differences in methods were of no particular concern. The soils at Wooster and Holgate were rather uniform; at the former a glaciated, noncalcareous silt loam, and at the latter, a lacustrine clay loam. At College Park, the soil was highly variable but, fortunately, duplicate plots were available there. The soil was silt loam derived from coastal-plain deposits.

The fall-protection treatments were applied to the plots between October 15 and 23, 1947. Three adjoining areas, each approximately 6 feet long and 4 feet wide, were selected in each plot. The center one of the three areas was left uncovered, while the outer ones were covered. One was covered with hay mulch about 1-inch thick. The other was covered with strips of black asphalt roofing paper 6 inches wide with 2 inches between strips. The latter material was used in order to compare a winter covering that was relatively undecomposable with the hay mulch. However, the paper covering turned out to be

very much less protective than the mulch. In addition, the paper was blown off the plots at Holgate by a cyclone that struck during the winter. Hence no data are shown in the results for the paper covering at Holgate. The cyclone also disturbed the mulch treatment on the continuous corn plot that received organic matter. Hence, data are not given for this plot either.

The following spring, between April 5 and 29, 1948, the plots were revisited. The earthworms were counted to a depth of 8 inches on squares measuring 7 by 7 inches. Samplings were in triplicate at College Park and Wooster, and in duplicate at Holgate. Infiltration was determined in triplicate by a previously described method (7). Soil samples were taken for duplicate water-stability tests. This procedure likewise has been described elsewhere (2).

The effects of the treatments on soil porosity were also determined. Soil cores were taken with a sampler similar to that described by Baver (1), but of improved design to minimize structural disturbances in the soil. The cores were placed in water overnight and then clamped on a tension plate. Water was introduced from the bottom until free water appeared at the soil surface. A 25-cm. tension was then drawn for 10 minutes. The amount of water that drained was considered a measure of the pore space through which free water drained readily. The tension of 25 cm. was imposed by the design of the equipment.

RESULTS

All the data for each location are given in table 1. Since the purpose of the study was to determine effects that are broadly applicable, the results will not be discussed separately for each location. Instead, they are summarized for all locations in table 2.

Owing to the lack of some of the Holgate values, the actual averages might have been adjusted in order to make them exactly comparable. However, such adjustments would necessarily have to be somewhat theoretical and since they were unnecessary to show the main effects, they were avoided.

The various soil-structural factors (infiltration, large pores, and water-stability) and the earthworm populations showed similar trends in the response to the covering treatments. Both the paper and mulch covers affected the observed factors in the same direction, but the effect was usually greater with the mulch than with the paper.

The plots in continuous corn showed a large increase in all factors as a result of the winter covering. Where the soil was not covered over winter, the plots from which stover had been removed gave slightly lower values than the plots that had received stover or manure.

The plots that had been in rotation corn during 1947 and had young wheat on them over winter likewise showed large increases in all factors as a result of the winter covering. Where the soil was uncovered over winter, the values were not quite as low in these plots as in the continuous corn plots.

The plots that had been in rotation wheat during 1947 and had a sod cover over winter showed some increase in infiltration, large pores, and earthworms, but not in water stability as a result of the winter covering. In plots uncovered over winter, all values were larger than in those that had been in corn in 1947.

TABLE 1.—*Infiltration rate, large pores, and water-stability of the soil and the earthworm population in April 1948, on agronomic plots given various overwinter protection treatments*

Cropping practice 1947	Location [1]	Overwinter protection		
		None	Paper	Mulch
Infiltration rate (in./min.): [2]				
Continuous corn	College Park	0 02	0 66	0 70
	Wooster	00	02	.11
	Holgate	.04		.90
Continuous corn Organic matter [3]	College Park	06	.31	31
	Wooster	.01	.00	.43
Corn in rotation	College Park	07	.49	.30
	Wooster	.02	.10	.17
	Holgate	.22		.46
Wheat in rotation	College Park	.08	.14	.04
	Wooster	.11	.23	.08
	Holgate	.15		.50
Continuous hay	College Park	38	.52	.94
	Wooster	58	.10	.35
	Holgate	.13		.17
Large Pores (percent): [4]				
Continuous corn	College Park	4.0	8, 1	7.9
	Wooster	1.0	2, 2	3.2
	Holgate	3.3		8.9
Continuous corn, organic matter [6]	College Park	4.2	9.7	12.5
	Wooster	2 2	3 2	5.8
Corn in rotation	College Park	4.2	9.4	8.0
	Wooster	2 8	7.6	7 6
	Holgate	4.8		5.4
Wheat in rotation	College Park	7.4	8.4	7.8
	Wooster	4 4	8.8	8.0
	Holgate	6 9		9.3
Continuous hay	College Park	12.6	6 6	9.0
	Wooster	8.2	9.0	9.6
	Holgate	10.7		5.9
Water-stability (percent): [5]				
Continuous corn	College Park	35 2	33. 6	41.6
	Wooster	12.6	14.3	14.0
	Holgate	41.0		48.5
Continuous corn, organic matter [6]	College Park	42.3	29.3	35.5
	Wooster	12.4	16 4	15 8
Corn in rotation	College Park	35.5	33.7	54.7
	Wooster	32.8	42.6	55 4
	Holgate	63.2		67 0
Wheat in rotation	College Park	68.4	57.5	59.6
	Wooster	58.8	51.6	57.6
	Holgate	78.0		80.9
Continuous hay	College Park	80.4	83.6	89 1
	Wooster	70.3	83.9	72.2
	Holgate	85 8		84.7
Earthworm population (No./sq. ft.): [6]				
Continuous corn	College Park	1.5	2.9	43.6
	Wooster	2.0	6.9	19.6
	Holgate	4.9		63.7
Continuous corn, organic matter: [6]	College Park	2.9	5.9	71.0
	Wooster	7.8	11.8	13.7
Corn in rotation	College Park	6 4	6.9	70.1
	Wooster	7 8	16 7	52 9
	Holgate	14.7		32.3
Wheat in rotation	College Park	22.5	38 7	86.7
	Wooster	13.7	40.2	42.1
	Holgate	31.4		45.1
Continuous hay	College Park	25.5	30.4	45.6
	Wooster	8.8	9.8	4.9
	Holgate	38.2		100.0

[1] All plots duplicated at College Park; single at Wooster and Holgate.
[2] Each value is the average of 3 samples per plot.
[3] Data for this cropping practice available at College Park and Wooster only.
[4] Each value is the average of 2 samples per plot.
[5] Each value is the average of 2 samples per plot.
[6] Each value is the average of 3 samples per plot at College Park and Wooster; 2 at Holgate.

TABLE 2.—*Average effects of overwinter protection on soil physical factors and earthworms in April 1948*

Observation	1947 Crop	Natural winter cover	Overwinter protection		
			None	Paper	Mulch
Infiltration (inches per minute).	Continuous corn	Corn stubble	0 02	0.34	0.57
	Continuous corn, organic matter.	Corn stubble	.04	.16	.37
	Rotation corn	Young wheat	10	.30	.31
	Rotation wheat	Clover	.11	.18	.31
	Hay	Sod	.36	.31	.19
Large pores, drained at 25 cm. tension (per-cent).	Continuous corn	Corn stubble	2 8	5.2	6.7
	Continuous corn, organic matter.	Corn stubble	3.2	6 4	9.2
	Rotation corn	Young wheat	3 9	8.5	7 0
	Rotation wheat	Clover	6 2	8.6	8.4
	Hay	Sod	10.5	7.8	8 2
Water stability (per-cent).	Continuous corn	Corn stubble	29.6	29.6	34.7
	Continuous corn, organic matter.	Corn stubble	27 4	22.8	25.6
	Rotation corn	Young wheat	43 8	49.0	59.0
	Rotation wheat	Clover	68 4	58.7	66.0
	Hay	Sod	78 9	87.6	82.0
Earthworms (number per square foot).	Continuous corn	Corn stubble	2 8	4.9	42.3
	Continuous corn, organic matter.	Corn stubble	5 4	8.8	42.1
	Rotation corn	Young wheat	9 6	11.8	51.8
	Rotation wheat	Clover	22 5	39.4	58.0
	Hay	Sod	24.2	20.1	50.2

The hay plots were least affected by the winter covering. Where the soil was uncovered over winter, the values were relatively high in these plots as compared with those for plots that had been in corn in 1947.

DISCUSSION

Values obtained from the continuous hay land that had not been covered over winter may be taken as the standard for judging good soil conditions. Where left uncovered over winter, the land that had been clean-cultivated during the summer of 1947 was in very much poorer condition than the sod. Mulching the clean-cultivated land overcame much of the difference. Its infiltration and earthworm counts were as favorable as in the sod, and the large pore space was almost as good. The water-stability of the soil was still much less than that of the sod land, but there was a definite improvement due to mulching.

The roofing-paper cover also improved the soil structural conditions on the cultivated plots but not so much as the hay mulch. This was due to the poor insulative quality of the paper as compared with the mulch; also in part, perhaps, to the organic matter that became available as food for soil organisms in the case of the mulch and not of the paper.

Mulching over winter would be practical only in gardens and on a few types of intensively operated farms. For most soils where deterioration of soil structure over winter is an important problem, more practical methods of obtaining adequate protection will have to be devised. As shown in this study, the insulative protection of winter grain is insufficient to maintain soil structure and earthworms over winter.

SUMMARY

The effect of winter protection on the structure and earthworm population of soils was determined in plots at College Park, Md.; Wooster, Ohio; and Holgate, Ohio. The soil-structure measurements used were infiltration rate, water-stability, and volume of large pores.

Clean-cultivated land left bare over winter had poorer soil structure and a lower earthworm population than land protected by sod (continuous or after wheat in rotation).

The soil structure and earthworm population were very much improved in clean-cultivated land by protecting the ground surface over winter with an insulative cover.

Winter covering had only slight effect on the structure of soil in sod.

Hay mulch was more effective than asphalt roofing paper in maintaining the structure of clean-cultivated soil over winter. Where mulching is impractical, other methods of applying the principle of winter protection to clean-cultivated land will have to be devised.

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VARIABILITY AND CORRELATION IN A COTTON BREEDING PROGRAM [1]

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INTRODUCTION

The cotton breeder is always concerned with two factors: The variability of the many characters with which he is working and the correlation between these characters. His breeding material must vary enough to include at least the potentialities for all the desired characters as well as for all other favorable characters of yield and quality. As his work progresses, he narrows the variability of the essential characters of quality, such as fiber strength, length, uniformity, fineness, and maturity, and of certain characters of yield, such as lint percentage, lint index, and boll weight. Frequently, characters seem to be correlated. This may present a problem to the breeder; because if a desirable character is associated with an undesirable one, selection for one without the other is difficult.

This article reports the biometrical relationships of certain characters used in the cotton breeding program at the New Mexico Agricultural Experiment Station. The purpose in determining these relationships was to discover how the characters in the breeding material are related, and to measure, from year to year, any changes in correlation.

PREVIOUS WORK ON CORRELATION OF COTTON CHARACTERS

The relationships between characters in upland cotton have been studied by many workers. Those reported vary greatly according to the material used and the place where the cotton was grown.

The yield of lint cotton from each plant was found by Stroman (23) [2] to depend on weight of bolls, number of five-lock bolls, number of four-lock bolls, number of vegetative branches, number of fruiting branches, height of plant, and lint percentage. The multiple correlation between yield of lint per plant and the other characters varied, in the several lines studied, from 0.96 to 0.99. The partial correlation coefficients between the characters indicated that number of bolls, weight of bolls, and height of plant were more important to yield than were the other characters. The height-yield relationship was negative. The weight of boll was negatively related to

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number of bolls. Hancock (*8*) reported significant negative correlations between height of plant and both number of fruiting branches and number of bolls. Buie (*3*) reported a multiple correlation of 0.86 between first-picking yield and date of first flower, flowering period, boll period, and first open boll. Harrison (*9*) reported, in Durango cotton, coefficients involving boll period, lint length, lint index, number of seed per boll, and weight of seed. Most of his correlations were significant.

Kearney (*13*, *14*), Humphrey (*11*), Hodson (*10*), Dunlavy (*5*), Martin and Mason (*17*), Stroman (*22*), and Patel (*20*), have reported relationships involving yield characters and length of lint. Most of these relationships were negative and significant. Stroman (*24*) reported relationships in the F_3 generation of *G. hirsutum* $\times$ *G. barbadense* cross. The correlations, some of which were significant, varied greatly in the different lines. The characters used were lint percentage, lint index, boll weight, and length of lint. Some relationships involving fiber length and color in Indian cotton have been reported by Kearney (*14*) and Kottur (*16*). Nanjundayya and Ahmad (*19*) reported a relationship between mean strength and weight per unit length which varied from 0.735 to 0.835, and one between length and strength of fiber from -0.15 to -0.30 ($n=45$).

Gulati and Ahmad (*7*) studying maturity of cotton fibers in relation to other characters, found the following significant correlation coefficients:

Characters:	*r*
Mean fiber strength and percent mature hairs	0. 72
Mean fiber strength and percent half mature hairs	−. 46
Mean fiber strength and percent immature hairs	−. 71
Fiber weight per inch and percent mature hairs	. 59
Fiber weight per inch and percent immature hairs	−. 60
Neps in yarn and percent immaturity of fibers	. 81
Fiber maturity and spinning performance (long cotton)	. 56

Koshal and Ahmad (*15*), studying swollen fiber diameter, obtained the following coefficients:

Characters:	*r*
Swollen fiber diameter and fiber weight per inch	0. 746
Swollen fiber diameter and standard hair weight	. 839
Swollen fiber diameter and spinning value	−. 800

Conrad and Berkley (*4*) found a correlation of 0.754 between X-ray arc length and number of 60.0 skein strength; they also found a multiple correlation coefficient of 0.91 between X-ray arc length and characters of fiber length, fineness, and maturity.

Turner and Venkataraman (*29*) investigated the relationship of the raw fiber characters and the spinning value of two series of Indian cottons. The partial correlations they found indicated that three raw fiber characters—fiber length, fiber weight per inch, and fiber width—were associated with spinning value. One of the coefficients they reported between fiber length and yarn strength was 0.87. Underwood (*30*), working with Egyptian cotton, reported a correlation coefficient of 0.77 between these two characters. Ahmad and Navkal (*1*), working with Indian cotton, reported significant correlation coefficients between all of the following characters: Spinning

value, mean fiber length, fiber weight per inch, and fiber length irregularity.

Turner (*28*) has reported a coefficient of −0.305 between count strength product and mean fiber strength. Barrett (*2*) reported correlation coefficients of −0.41 and −0.61 between yarn strength and fiber strength. Hutchinson and Govande (*12*) found a significant coefficient of −0.58 between the highest standard count and swollen fiber diameter, and one of −0.597 between the highest standard count and hair weight.

Webb and his associates (*32, 33, 34, 35*) have, in the last few years, completed preliminary reports on the biometrical relationships of the raw fiber characters and spinning performance of American cotton. The raw fiber characters are: Strength of fiber, maturity of fiber, length variability, fineness, upper half mean or three-fourths point, and grade.

MATERIALS AND METHODS

The individuals composing the populations were a mixture of related families in the regular breeding program at the New Mexico Agricultural Experiment Station. The families originated from early Acala selections, from general fields, and from crosses between certain related strains. Some of the families may have been derived from natural hybrids of more or less closely related strains, but no wide-cross progenies were included. Each year, the population was limited to those individuals given complete laboratory tests.

The characters used to indicate lint yield were: Lint percentage, which is obtained by dividing the weight of the lint in a sample by the weight of the lint and the seed; lint index, which is the percentage of lint multiplied by the weight of 100 seeds and then divided by the percentage of seed; and boll weight, which is the average weight of lint in a boll, in grams, from a 15-boll sample. In 1937, two other characters were also used—total bolls per plant and total fibers, or the total weight of fibers in a sample as measured by the Pressley Fiber Sorter.

Characters indicating fiber length were: Classer's length, as determined by a licensed government classer; the percentage of fibers from a 10-seed sample which measures 1⅛ inches or more in length, and which will be referred to in this paper as percentage 1⅛-inch (plus) fibers; and the three-fourths point, determined by use of a Pressley Automatic Fiber Sorter, which also was used to determine the percentage of 1⅛-inch (plus) fibers and the mean length of lint.

Uniformity of lint length was determined by the standard deviation of all the fibers in a sample. The percentage of 1⅛-inch (plus) fibers is also a measure of uniformity.

Strength of fiber is measured on a Pressley Strength Tester. It is reported as an index number, derived by dividing the pounds required for breakage by the weight of the broken fibers, which are of a standard length. In 1944, the strength index-percentage of standard sample was used. It is obtained by breaking a standard sample in every fifth sample.

Fiber diameter is obtained by measuring from 50 to 100 fibers, which have been swollen in an 18-percent sodium hydroxide solution,

with a micrometer eyepiece in a microscope. The eyepiece and objectives used give readings which are two-thirds of the swollen fiber size, or approximately the natural diameter of the fiber.

The variability in fiber diameter is expressed by the standard deviation of 50 to 100 fibers measured for fiber diameter. This character was used only in the calculation of simple correlation coefficients in 1944.

Fiber maturity, which was also used only in 1944, was measured by swelling fibers in an 18-percent sodium hydroxide solution and counting the mature fibers in a 100-fiber sample.

The tables indicate which characters were used each year. The number of individuals composing the populations were as follows: 1937, 162; 1940, 151; 1944, 441; 1945, 143; and 1946, 313.

Simple correlation coefficients were calculated each year between the characters. From these coefficients, multiple correlation coefficients were calculated (*28*). The partial correlation coefficients between all characters were calculated by multiple regression coefficients (*34, 23, 6*).

THE DATA

VARIABILITY

The mean, standard deviation, and the coefficient of variability of all the characters used in this study are found in table 1. The variability data are included mainly to show levels of the characters used in calculating the correlation coefficients. In passing, however, it is well to note a few points that may be interesting from the standpoint of practical cotton breeding. It is shown that, in 1937, the mean lint percentage of the progenies was 39.99 percent; the lint index was 9.08; the classer's length of lint was 36.51 thirty-seconds inch; the boll weight was 3.27 grams of lint. These had been built up over a long period. During this time, the percentage of 1⅛-inch (plus) fibers had been built up by the breeding program to 37.07 percent. The first lines tested for this character ranged between about 5 percent to practically 30 percent. As the work progressed and the testing for percentage 1⅛-inch (plus) fibers became more extensive, progenies with higher and higher percentages were developed. The lint percentage, lint index, classer's length, and boll weight decreased somewhat through the period shown, but while this occurred, the percentage of 1⅛-inch (plus) fibers increased to 52.01 percent in 1946. The fiber diameter of 13.93 microns in 1940 was a desirable diameter, but in 1944 it was coarser, 15.42 microns, and in 1946 it had decreased nearly 1 micron.

From 1937 to 1946 the variability, as measured by the standard deviation and coefficient of variability, was recorded for most characters. However, the range of most characters still gave a rather wide amount of variation. In cotton breeding, the variability must be wide enough to indicate the expression of as many genes as possible and it can be narrowed only through the testing, hybridization, and selection of the desirable progenies and individuals. Because certain characters are influenced by seasonal conditions, the degree of improvement can be measured only over long periods.

TABLE 1.—*Mean, Standard Deviation, and Coefficient of Variation of Each Character Used Each Year in Calculation of Correlation Coefficients*

	1937	1940	1944	1945	1946
Means:					
Lint percentage	39.99	36.10	37.37	34.90	37 15
Lint index	9.08	8.25	8.28	7.81	8.26
Boll weight (grams)	3 27	3.14		2.94	2.93
Total fibers (milligrams)	353.70				
Total bolls per plant (number)	47.20				
Classer's length (1/32 inch)	36 51		35 63	36.17	35.95
Percentage 1⅛-inch (plus) fibers	37.07	43.44	50.68	46.38	52.01
Mean length (inches)				.99	1.02
Three-fourths point (inches)					1.13
Length variability				25.32	23.88
Strength index			7.12	6.61	6.80
Strength index percent standard			102.08		
Fiber diameter (microns)		13.93	15.42	14.82	14.46
Standard deviation of fiber diameter			2.76		
Fiber maturity (percentage)			91.24		
Standard deviations:					
Lint percentage	2.22	1.78	1.93	1.82	1.59
Lint index	.91	.69	.64	.69	.70
Boll weight	.38	.34		.51	.29
Total fibers	49.06				
Total bolls per plant	13.79				
Classer's length	.80		1.25	.68	.78
Percentage 1⅛-inch (plus) fibers	9.46	12.00	13.71	10.92	11.39
Mean length				.04	.05
Three-fourths point					.05
Length variability				2.19	2.39
Strength index			.99	.51	.56
Strength index percentage standard			7.59		
Fiber diameter		.61	.92	.55	.54
Standard deviation of fiber diameter			.54		
Fiber maturity			5.19		
Coefficients of variability (percent):					
Lint percentage	5 5	4.93	5.18	5.22	4.29
Lint index	10 1	8.36	7.79	8.79	8.53
Boll weight	11.6	10.83		17.29	9.86
Total fibers	13.9				
Total bolls per plant	29 2				
Classer's length	2.2		3.51	1.89	2.16
Percentage 1⅛-inch (plus) fibers	25.5	27.62	27.05	23.54	21.90
Mean length				4.08	4.47
Three-fourths point					4.43
Length variability				8.64	9.99
Strength index			13.84	7.78	8.19
Strength index percentage standard			7.43		
Fiber diameter		4.31	5.96	3.73	3.71
Standard deviation of fiber diameter			19.46		
Fiber maturity			5.68		
Populations	162	151	441	143	313

RELATIONSHIPS OF THE CHARACTERS

Tables 2 to 7, inclusive, show the simple and partial correlations between all possible combinations of characters. The discussion is limited to the partial correlations, because these show the relationship between two characters and eliminate the influence of the fluctuations of the other characters.

The data show that the relationship of certain characters may not be the same each year. Different climatic and soil conditions of the different years certainly influence the expression of certain genes. Selection also contributes some influence on the relationships. However, certain correlations remain about the same from year to year.

Characters of Yield

Lint percentage and lint index are strongly correlated each year.

The classic example of correlation in cotton characters, the negative correlation between lint percentage and length of lint, occurred

TABLE 2.—*Simple (S) and Partial (P) Correlation Coefficients of Lint Percentage With Other Characters*

Characters	1937		1940		1944		1945		1946	
	S	P	S	P	S	P	S	P	S	P
Lint index	0.76	0 61	0 61	0.49	0 38	0.46	0.69	0.64	0 59	0 56
Percentage of 1⅛-inch (plus) fibers	−.36	−.32	−.44	−.35	−.57	−.44	−.02	−.02	−.21	−05
Classer's length lint	.16	−02			−.34	−.14	−.02	−14	−22	−.14
Strength index					−.37	−.25	−.21	−.09	−24	−.18
Fiber diameter			.10	−.12	−.03	−.06	.22	.06	14	−004
Mean length lint							−10	.01	−18	−.08
Lint length variability							19	.12	.02	−.04
Boll weight	60	.13	.42	.09			.43	.34	.26	.001
¾ point									−.17	.06
Total fibers	.19	−25								
Least significant *r:*										
5-percent level	.15	.16	.16	.16	.09	.09	.16	.17	.11	.11
1-percent level	.20	.20	.21	.21	.12	.12	.21	.22	.14	.15

TABLE 3.—*Simple (S) and Partial (P) Correlation Coefficients of Lint Index With Other Characters*

Characters	1937		1940		1944		1945		1946	
	S	P	S	P	S	P	S	P	S	P
Lint percentage	0 76	0.61	0 61	0.49	0.38	0.46	0.69	0.64	0 59	0.56
Percentage of 1⅛-inch (plus) fibers	−19	.10	−.30	.05	−.04	.11	.16	.13	.04	−02
Classer's length unit	.19	.10			.05	.04	.14	.17	−.04	.02
Strength index					.17	.19	−.14	−.05	−.11	.04
Fiber diameter			.19	.11	.32	.20	.17	.06	.20	.11
Mean length lint							.11	−.09	.09	.15
Lint length variability							03	−.13	−.11	.05
Boll weight	.73	.43	.53	.37			.34	.01	.45	.35
¾ point									.04	−09
Total fibers	.45	.37								
Least significant *r:*										
5-percent level	.15	.16	.16	.16	.09	.09	.16	.17	.11	.11
1-percent level	.20	.20	.21	.21	.12	.12	.21	.22	.14	.15

TABLE 4.—*Simple (S) and Partial (P) Correlation Coefficients of Percentage of 1⅛ + Fibers With Other Characters*

Characters	1937		1940		1944		1945		1946	
	S	P	S	P	S	P	S	P	S	P
Lint percentage	−0.36	−0 32	−0.44	−0.35	−0.57	−0.44	−0.02	−02	−0 21	0.05
Lint index	−19	.10			−.04	.11	.16	.13	.04	−.02
Classer's length lint	−.05	−.01			.46	.27	.31	−.60	.39	.04
Strength index					.43	.20	.26	.04	.17	.08
Fiber diameter			−.27	−.24	.18	−.05	−.13	.10	−.02	.04
Mean length lint							.90	.96	.91	.57
Lint length variability							−.20	.81	−.26	.17
Boll weight	−.17	−.002	−.30	−.11			.01	.04	.07	.02
¾ point									.87	.26
Total fibers	−.01	.01								
Least significant *r:*										
5-percent level	.15	.16	.16	.16	.09	.09	.16	.17	.11	.11
1-percent level	.20	.20	.21	.21	.12	.12	.21	.22	.14	.15

TABLE 5.—*Simple (S) and Partial (P) Correlation Coefficients of Classer's Length of Lint With Other Characters*

Characters	1937		1944		1945		1946	
	S	P	S	P	S	P	S	P
Lint percentage	0 16	−0 02	−0 34	−0 14	−0 02	−0 14	−0 22	−0.14
Lint index	.19	10	.05	.04	14	.17	− 04	.02
Percentage of 1⅛-inch (plus) fibers	− 05	−.01	46	.27	.31	−.60	39	04
Strength index			34	06	13	.002	05	−.05
Fiber diameter			.34	.20	− 15	− 15	− 03	− 01
Mean length lint					.45	.68	39	06
Lint length variability					− 07	.61	− 12	.001
Boll weight	17	05			21	22	− 004	− 004
¾ point							.37	04
Total fibers	.03	− 07						
Least significant *r:*								
5-percent level	.15	.16	.09	09	.16	.17	.11	.11
1-percent level	.20	.20	.12	.12	21	.22	.14	.15

TABLE 6.—*Simple (S) and Partial (P) Correlation Coefficients of Strength Index With Other Characters*

Characters	1944		1945		1946	
	S	P	S	P	S	P
Lint percentage	−0 37	−0.25	−0.21	−0.09	−0.24	−0.18
Lint index	.17	.19	− 14	−.05	−.11	.04
Percentage of 1⅛-inch (plus) fibers	43	20	26	.04	.17	.08
Classer's length lint	34	06	.13	.002	.05	−.05
Fiber diameter	.45	41	−.31	−.24	− 14	−.12
Mean length lint			.27	.02	15	−.04
Lint length variability			−.13	.01	− 09	−.06
Boll weight			− 03	.05	−.07	−.02
¾ point					13	.01
Least significant *r:*						
5-percent level	.09	09	.16	.17	.11	.11
1-percent level	.12	.12	21	.22	.14	.15

TABLE 7.—*Simple and Partial Correlation Coefficients of Fiber Diameter With Other Characters*

Characters	1940		1944		1945		1946	
	S	P	S	P	S	P	S	P
Lint percentage	0.10	−0.12	−0.03	0.06	0 22	0.06	0 14	−0 004
Lint index	.19	11	.32	.20	.17	.06	.20	.11
Percentage of 1⅛-inch (plus) fibers	−.27	− 24	18	−.05	−.13	.10	−.02	.04
Classer's length lint			.34	.20	−.15	−.15	−.03	−.01
Strength index			45	.41	−.31	−.24	−.14	−.12
Mean length lint					−.18	.10	−.03	−.11
Lint length variability					.16	.13	− 01	− 08
Boll weight	.21	.10			04	− 001	.18	.09
¾ point							.0003	.08
Least significant *r:*								
5-percent level	.16	.16	.09	.09	.16	.17	.11	.11
1-percent level	.21	21	.12	.12	21	.22	.14	.15

only twice when length of lint was measured by classer's length, mean length, or three-fourths point. The partial correlation coefficient between lint percentage and classer's length was significant in 1944 and 1946. When length was measured by percentage of 1⅛-inch (plus) fibers, which is more a measure of fiber length uniformity than of length, the partial negative correlation between fiber length and percentage was significant in 1937, 1940, and 1944, but not in 1945 and 1946.

This may indicate that, as the individuals with a high percentage of lint and a high percentage of 1⅛-inch (plus) fibers were selected along with the other desirable characters, the correlation has been broken, and that the genes of both lint percentage and percentage of 1⅛-inch (plus) fibers in the breeding material in 1945 and 1946 were independent.

In 3 of the 4 years when boll weight was tested, the positive partial correlation cofficients between it and lint index were significant. Outside of that and two other correlations, boll weight is independent of the other characters, according to the data. In one population, it was correlated with classer's length; in another, with lint percentage.

Characters of Lint Length

The mean length of lint and lint length variability were used only in 1945 and 1946. They were correlated with each other and with the percentage of 1⅛-inch (plus) fibers.

Percentage of 1⅛-inch (plus) fibers was also correlated, in 1946, with the three-fourths point. In 1944, this character was positively correlated with the classer's length, but in 1945, the two were negatively correlated. The 1945 relationship is not easily explained, because the percentage of 1⅛-inch (plus) fibers should be positively correlated with classer's length. Classer's length is influenced by fiber strength as well as length, and in 1945, the classer's length may have been based on a finding that the longer fibers were weaker than the shorter fibers. But if this were the case it was not confirmed by a correlation between percentage of 1⅛-inch (plus) fibers and strength index.

In 1946, the classer's length was not associated significantly with any of the other characters, as shown by the partial correlation coefficients in table 5. The same table shows that, in 1945, the character was negatively correlated with percentage of 1⅛-inch (plus) fibers, and positively with mean length, length variability, and boll weight. In 1944, it was associated with percentage of 1⅛-inch (plus) fibers and with fiber diameter.

Strength Index

Table 6 shows that the strength index was not strongly correlated with any of the characters used. Significant, though small, negative correlations were found between this character and lint percentage in 1944 and 1946. It was positively correlated with fiber diameter in 1944 and negatively in 1945.

Fiber Diameter

There were no significant partial correlation coefficients between fiber diameter and the other characters in 1946, as shown in table 7. In 1945, fiber diameter and strength index were negatively correlated,

and in 1944, they were positively correlated. The character was negatively correlated with percentage of 1⅛-inch (plus) fibers in 1940, but not in other years. In 1944, it was positively correlated with lint index and classer's length.

<div align="center">MULTIPLE CORRELATION COEFFICIENTS</div>

Table 8 shows the multiple correlation for all the characters used each year. None of the coefficients are very large. This is an encouraging sign for the breeder, because strong associations, where some are positive and some are negative, are obstacles in the breeding program.

<div align="center">DISCUSSION</div>

The data show that correlations between the characters studied in the cotton breeding material were not always consistent from one year to another. If a generalization may be drawn from this study, it is that no correlations are so strong as to preclude unaccounted-for variation. By utilizing this variation, the cotton breeder should be able to combine all the desirable characters found in the material at hand.

TABLE 8.—*The multiple correlation coefficients (R) and coefficient of determination (R²) involving various characters*

Characters	1937		1940		1944		1945		1946	
	R	R²	R	R²	R	R²	R	R²	R	R²
Lint percentage	0.80	0.65	0.68	0.46	0 71	0.51	0.76	0 58	0.66	0.44
Lint index	.86	.74	.69	.48	.55	.31	.72	.53	.69	.48
Percentage of 1⅛-inch (plus) fibers	.38	.14	.50	.25	67	.45	.97	.94	.93	.87
Classer's length lint	.21	.04			.55	.31	.75	.56	.43	.18
Strength index					.67	.44	.41	.17	.31	.09
Strength index percentage standard					.41	.17				
Fiber diameter			.33	.11	.60	.36	.39	.16	.28	.08
Mean length lint							.98	.96	.96	.92
Lint length variability							.88	.78	.74	.55
Boll weight	.74	.54	.56	.32			.50	.25	.47	.22
¾ point									.92	.85
Total fibers	.53	.28								
Total bolls per plant	.23	.05								

Correlation coefficients show a relationship between two characters. Each of these characters may be the result of the expression of several genes. Significant correlations between characters may be caused by multiple factors, some of which may influence both characters in combination with other genes. Also, one or more of the genes involved in the expression of one character might be linked with one or more of the genes which cause the expression of the other character. The relationships given here represent the available material of the breeding program for each year. The genotypes involved in 1 year therefore may be different from the genotypes involved for the other years. Furthermore, genes express themselves according to their environment, which might cause relationships measured by the correlation coefficient to be different from year to year. Unpublished data at this station destroyed by fire in 1937, indicated that variety, soil, fertilizer treatment, and irrigation treatments influence the relationship of the characters of the cotton plant.

SUMMARY

The simple, partial, and multiple correlation coefficients were determined between characters in the cotton breeding material of the New Mexico Agricultural Experiment Station for 5 years. These characters were: Lint percentage, lint index, boll weight, classer's length, percentage of 1⅛-inch (plus) fibers, mean length, length variability, three fourths point, strength index, and fiber diameter. The material used was related selected progenies and these were given the complete laboratory tests.

Lint percentage and lint index were positively correlated each year. Other significant correlations varied as to degree and relationship for the different years.

The wide variability, even with the correlations, offers the possibility of combining all the desired characters of yield and quality into one strain. This necessitates the use of a larger number of individuals from selected line-bred or hybrid material.

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FUSARIA ASSOCIATED WITH MIMOSA WILT, SUMAC WILT, AND PINE PITCH CANKER [1]

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INTRODUCTION

In recent years several new tree diseases caused by species of *Fusarium* have been described. At times other Fusaria in addition to the species responsible for the diseases have been found closely associated with the pathogens. The Asheville, N. C., laboratory of the Division of Forest Pathology has worked with 6 of the 10 species of *Fusarium* recognized by Snyder and Hansen (*13, 14, 16*) [4] and with a number of formae in connection with studies on the mimosa wilt (*5, 18*), the sumac wilt (*20*), and the pitch canker disease of pine (*8*). This paper is intended to clarify the taxonomy and nomenclature of the Fusaria encountered in studying these 3 diseases and presents information of value in making identifications and in understanding the variability encountered in natural clones [5] and laboratory mutants within a given species.

In the final assignment of names to the various species studied in this work, the system of classification and nomenclature of Snyder and Hansen (*13, 14, 16*) is used. In this system the species is based upon morphology and the forma in a trinomial denotes pathogenicity to a certain host or hosts. The extreme variability within some species, and even within some formae, with regard to spore size, pigmentation, conidial formation and septation, and gross cultural appearance among natural clones as well as subsequent mutants and among cultures exposed to different environmental conditions requires adoption of such a system if an endless number of species names—one applied to each variant—is to be avoided. Since this wide range of variation can be obtained among clones from single-spore cultures derived from a single pure culture of an isolate from nature, the naming of such

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[3] In cooperation with Southeastern Forest Experiment Station, U. S. Forest Service, Asheville, N. C.

[4] Italic numbers in parentheses refer to Literature Cited, p. 381.

[5] The term "natural clone" denotes any form isolated directly from nature and is synonymous with Miller's "wild type" (*9*) where the latter is used in this sense. The term is used in opposition to "laboratory clone," the latter denoting mutants or growth types which develop in the course of laboratory culture practice.

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(365)

variants as separate species is untenable. No spore measurements for the three formae being named are given in this paper, because their spore dimensions fall within the common ranges given by Wollenweber and Reinking (*22*) for the respective species.

IDENTIFICATION IN FUSARIUM

Species identification of an unknown *Fusarium* required that its morphologic features be determined, since these should constitute the sole basis on which the species are recognized. Since the sporulation of a *Fusarium* in nature is usually in the form of sporodochia, the attempt to determine *Fusarium* species directly from field specimens necessarily restricts a morphologic study largely to the sporodochia themselves. Often the macroconidia so obtained in nature reflect unfavorable influences of environment, substrate, other competitive organisms, and undermaturity or overmaturity. Furthermore, in nature more than one *Fusarium* species may invade a given substrate and form reproductive organs on the same tissues. Because of these facts, it is imperative that all efforts to identify *Fusarium* species be preceded by their transfer to a suitable culture medium by the single-spore method and that they be cultivated under conditions best suited to the development of the reproductive structures used in taxonomy.

PURE-CULTURE METHOD

Every culture referred to in this paper was initiated by a single spore transferred to a potato-dextrose-agar slant. Single-spore cultures were prepared either directly from spores found in nature, for example, on bark, or from conidia produced after a few days on tissue platings made from invaded plant parts. Ordinarily 10 single-spore cultures were prepared from each specimen; but, if the supply of conidia was inadequate, single-hyphal-tip cultures were prepared instead. Dilution plates of spores and of hyphal fragments were made on water agar.

During the summer and fall the cultures were laid on tables near a window, where they were exposed during their entire development to diffuse but not direct sunlight. Under these conditions most of the Fusaria included in this study sporulated normally; however, in some cases it was necessary to incubate the cultures outdoors, where they were subject not only to fluctuating daylight but also to widely fluctuating temperatures (*15*). Only in this way, for example, was there obtained an abundance of well-developed sporodochia of the pitch canker *Fusarium* from pine.

THE FUSARIA ON ALBIZZIA

The demonstration, by Hepting (*5*) and Toole (*18*) in the United States and later by Campi (*2*) in Argentina, of a vascular fusarium wilt of the mimosa tree (*Albizzia julibrissin* Durazz.) and the description by Voronikhin (*21*) of what is probably the same disease in the

Soviet Union have made important the correct identification of the various species of *Fusarium*, some saprogenic, occurring on the mimosa tree.

Culture studies and inoculations have demonstrated that the mimosa wilt *Fusarium* in the United States and Argentina is a form of *F. oxysporum*. In the following discussion the names that have correctly or mistakenly been applied to this fungus are taken up in order of their appearance in the literature.

Fusarium albizziae Voron. (*Nectria albizziae* Voron.)

In 1920, Voronikhin (*21*) described a disease of the mimosa tree, epidemic in the Soviet Union, that appears to agree in all respects with that later described in the United States by Hepting (*5*). Although Voronikhin demonstrated the presence of microconidia in the infected vessels and observed cushions of *Fusarium* macroconidia on the surface of shoots killed by the wilt, he apparently made no cultures of the fungus and no inoculations. Nor did Voronikhin present any evidence to show whether the *Fusarium* found on the bark was the same fungus as that seen in the vascular tissue or even whether it was pathogenic. Without proof of the connection between the surface *Fusarium*, which he described as *F. albizziae*, and his vascular pathogen, this name cannot be accepted as that of the causal agent of mimosa wilt. Voronikhin's descriptions indicate strongly that the bark *Fusarium* was not of the wilt (*oxysporum*) type.

Perithecia found on a branch of mimosa killed by wilt were described by Voronikhin as *Nectria albizziae*, on the basis of their association with the *Fusarium* also found fruiting on the bark. No proof of genetic relationship to the *Fusarium* was given, and therefore there is no certainty that the ascomycete was the same fungus. The perithecia were small and protruded in a group on a cushionlike stroma. No wilt Fusaria are known to produce perithecia, and thus additional doubt is attached to Voronikhin's conclusions about the connection of this fungus with the vascular parasite.

Fusarium perniciosum Hepting

Proof that the pathogen of the vascular wilt of mimosa is a *Fusarium* was first established by Hepting (*5*), who also noted the resemblance of the fungus he isolated to other vascular wilt Fusaria. Partly because of its selective pathogenicity for the mimosa tree and partly because of the short macroconidia in his cultures (mean length of 28μ for three-septate spores) Hepting named his fungus *F. perniciosum*, in keeping with the Wollenweber system of *Fusarium* classification. Hepting (*5*) and Toole (*18*) also mentioned clones differing widely from the common one in spore size and septation and in gross cultural appearance. These clones are now recognized merely as morphologic variants of the wilt fungus.

Fusarium oxysporum f. *perniciosum* (Hepting) Toole

This name was first suggested in a footnote by Toole (*18*) in 1941, but it was not proposed.

Fusarium oxysporum f. *perniciosum* (Hepting) Snyder

In 1943 Campi (*2*) published a paper in which the trinomial *F. oxysporum* f. *perniciosum* (Hepting) Snyder was used without Snyder's knowledge. Although Campi cited Toole's paper, either she did not see his footnote or she did not consider Toole's trinomial as valid under the rules, which state that the name of a taxonomic group is not validly published unless it is definitely accepted by the author who publishes it.

Campi's trinomial, however, also comes into question, since she did not definitely propose the combination but stated that *F. perniciosum*, according to the latest classification of Snyder, would be called *F. oxysporum* Schlecht. f. *perniciosum* (Hepting) Snyder.

NAME ACCEPTED FOR THE WILT FUSARIUM

In view of the confusion which exists in the nomenclature of the mimosa wilt pathogen, it seems desirable that a name validly published and still in accord with the trinomial system of *Fusarium* taxonomy (*13*, *14*, *16*) be indicated for acceptance.

It is proposed that the name *F. perniciosum* be changed, as suggested by Toole (*18*), in conformance with the *Fusarium* classification of Snyder and Hansen (*13*) as follows:

Fusarium oxysporum Schlecht. emend. Snyder and Hansen forma *perniciosum* (Hepting) Toole.

Syn. *F. perniciosum* Hepting (*5*).
F. oxysporum f. *perniciosum* (Hepting) Snyder (*2*).

A form of the species *F. oxysporum* pathogenic in the vascular tissues and causing a wilt in *Albizzia julibrissin* and by inoculation in *A. lebbeck* (L.) Benth., *A. lophantha* Benth., and *A. kalkora* (Roxb.) Prain (*18*). In southeastern United States, Argentina, and probably the Soviet Union.

ISOLATION OF FUSARIUM OXYSPORUM F. PERNICIOSUM

The mimosa wilt *Fusarium* may be obtained in culture by plating either the discolored vascular tissues of a diseased tree or the sporodochia which sometimes push out from the bark lenticels of a wilted tree when the environment is very humid. Sporodochia of some saprophytic Fusaria, however, occur even more commonly on the bark of dead or dying mimosa. Cultures made from sporodochia on the bark and from discolored wood beneath it have yielded identical clones of *F. oxysporum* f. *perniciosum* on several occasions, as shown by inoculation tests.

Hepting (*5*) found that several species of *Fusarium* may be recovered from mimosa bark and that at least one, which he recorded as *F. solani* var. *martii* (Appel and Wr.) Wr., could be isolated occasionally from diseased wood. This variety, synonymous with *F. solani* (Mart.) Appel and Wr. emend. Snyder and Hansen, was found to be unable to attack mimosa by itself (*5*); yet in nature it follows closely the wilt pathogen. This finding has recently been confirmed. Not only has *F. solani*, hidden in mixed cultures with *F. oxysporum* f. *perniciosum*,

been recovered from tissue platings of the discolored vascular region of wilted mimosa trees, but also the perfect stage, *Hypomyces solani* Reinke and Berth. emend. Snyder and Hansen, has been collected on the bark in association with sporodochia of *F. oxysporum* f. *perniciosum*. In fact, numbers of perithecia have been found in such cases clustered around sporodochia of the wilt *Fusarium*, an association which on the basis of appearances might well be considered to be that of a *Fusarium* and its *Nectria* or *Hypomyces* ascigerous stage. Only by making single-ascospore cultures from the perithecia and single-conidium cultures from the sporodochium in each case was the association detected. Perithecia of *Hypomyces* later developed in the single-ascospore cultures, especially in those incubated outdoors, demonstrating with certainty in these instances not only the identity of the fungus but also the fact that it was homothallic. Both heterothallic and homothallic strains of *H. solani* are known.

VARIABILITY IN FUSARIUM OXYSPORUM F. PERNICIOSUM

Although the use of natural clones is favored for the purpose of identification, it should be emphasized that natural clones may differ considerably among themselves (fig. 1). Some natural clones of the mimosa *Fusarium* produce abundant mycelium with but few slowly formed sporodochia; others form sporodochia rapidly and in abundance; still others are intermediate in appearance or differ in pigmentation from white to salmon to vinaceous shades or in their ability to develop sclerotia. Macroconidia may agree in size with those earlier described by Hepting for *F. perniciosum* (as in fig. 1, *C*), may be much larger (fig. 1, *D*) as in Hepting's Wisacky clone, or may otherwise reflect the variability in morphology displayed so commonly in the species *F. oxysporum*.

Selected natural clones, some of their variants, and their spores are shown for the mimosa wilt *Fusarium* in figure 1. The diverse clones shown here represent about 50 isolates made during the 1947 season, from different localities and States.

As an aid in recognizing the variability within this forma, the more common clones are described briefly as follows:

Clone A.—Commonest clone (fig. 1, *A*, last six tubes). Thallus raised; white, pink, or vinaceous shades; macroconidia rarely more than three-septate, produced abundantly in sporodochia and on surface of mycelium, salmon-colored in mass; microconidia abundant; no sclerotia, but often plectenchymic masses.

Clone B.—Hepting's Wisacky clone (fig. 1, *A*, middle three tubes). Thallus raised; white to vinaceous shades; macroconidia abundant, commonly four- or five-septate, produced in sporodochia and on surface of mycelium; microconidia abundant; blue-black sclerotia common.

Clone C.—Like clone A, but no sporodochia or plectenchymic masses (fig. 1, *A*, first three tubes, fig. 1, and *B*, fourth three tubes).

Clone D.—Pionnotal clone (fig. 1, *B*, first three tubes). No aerial mycelium; slimy spore mass over agar, mostly one- to three-septate macroconidia; some microconidia; color whitish to buff, pink or vinaceous; no sporodochia, plectenchymic masses, or sclerotia.
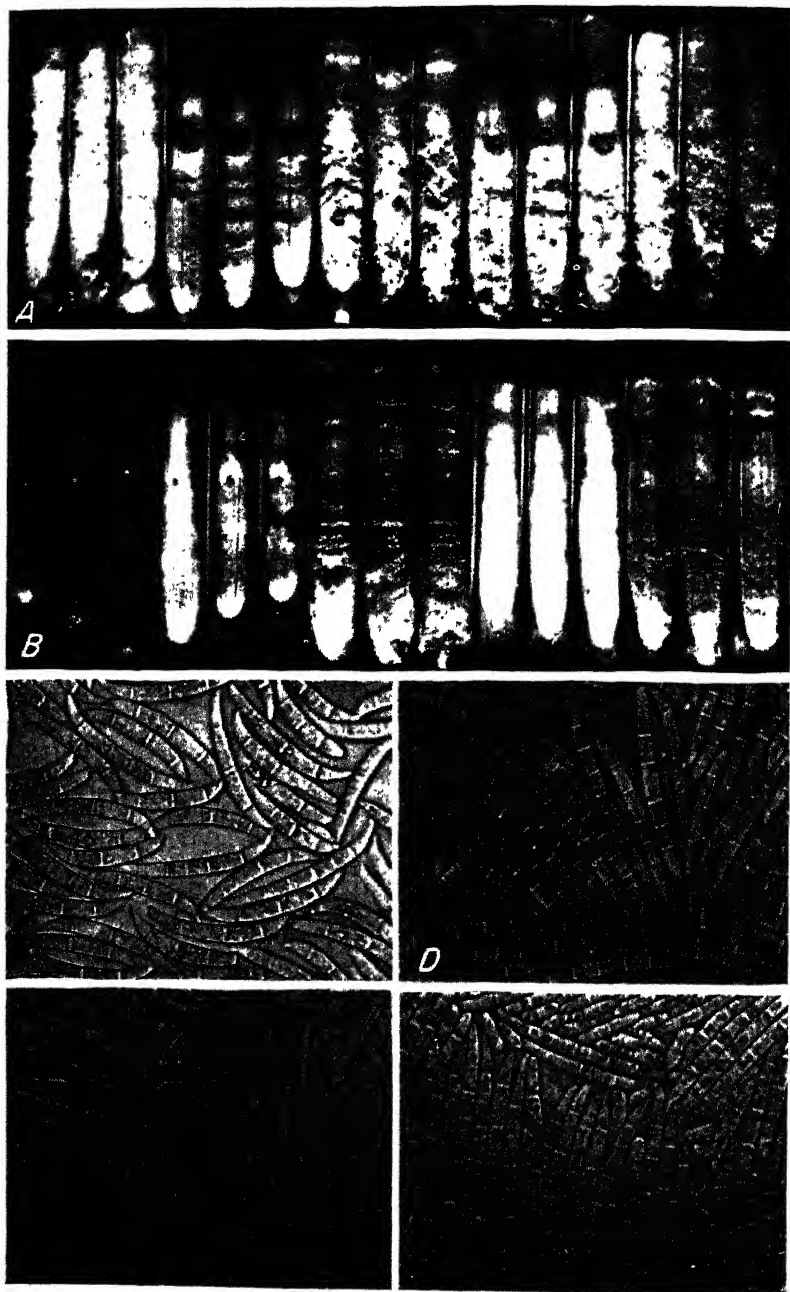
FIGURE 1.—*Fusarium oxysporum* f. *perniciosum.* *A* and *B,* Triplicate single-spore cultures of representative clones, made the same day on potato-dextrose agar: *A,* Identical with clones isolated directly from nature; *B,* laboratory mutants. *C,* Average-sized macroconidia from culture kept indoors. *D,* Long macroconidia from cultures made the same day from the same culture as those in *C* but kept outdoors. *E,* Very short macroconidia from a clone grown indoors. *F,* Long macroconidia from another clone grown indoors during the same period as those shown in *C, D,* and *E.* *C–F,* × 500,

<div align="center">FUNGI ASSOCIATED WITH MIMOSA WILT</div>

The Hyphomycetes and Ascomycetes commonly found on the bark of wilted mimosa in this investigation may be characterized briefly as follows:

Fusarium oxysporum f. *perniciosum*

Flesh-colored sporodochia, composed mostly of macroconidia, protruding from lenticels. In culture: pigmentation and cultural pattern various (see p. 370). Microconidia; macroconidia three- to five-septate, usually widest in the upper third; terminal and intercalary chlamydospores in the mycelium and spores.

Cultures of a form of *F. oxysporum* isolated from discolored xylem of a dying tree of *Albizzia procera* (Roxb.) Benth. in Puerto Rico failed to induce wilt in *A. julibrissin* in a small-scale test.

Fusarium solani (*Hypomyces solani*)

Cream, green, or blue (sometimes turning brown with age) sporodochia of macroconidia on bark or protruding from lenticels. In culture: cream, green, or blue pigmentation associated with spore masses, but mycelium usually not pigmented. Microconidia; terminal and intercalary chlamydospores in the mycelium and spores; macroconidia three- to five-septate or more, curved-cylindrical, apical cells usually blunt.

Ascigerous stage a *Hypomyces;* perithecia red, usually in groups around a sporodochium; ascospores hyaline, one-septate, usually tan-colored in mass.

Fusarium decemcellulare Brick (*Calonectria rigidiuscula* (Berk. and Br.) Sacc.)

Cream-colored spore masses inconspicuous, small, on bark. In culture: pigmentation next to agar usually carmine red, aerial mycelium white. Microconidia in chains, conspicuous; chlamydospores lacking; macroconidia in creamy masses, very large, 5- to 11-septate, curved-cylindrical as in *F. solani*.

The ascigerous stage was not observed on bark. Perithecia yellow to brown; ascospores hyaline, curved, usually three-septate, tan-colored in mass.

Fusarium episphaeria Snyder and Hansen (*Nectria episphaeria* Tode ex Fr.)

Conidial stage on bark inconspicuous or a flesh-colored slime. In culture: very slow-growing colonies, salmon to pink or orange-colored, consisting mostly of prostrate mycelium covered with a conidial slime, aerial mycelium usually absent. Microconidia usually absent, chlamydospores present or absent, macroconidia long, slender, one- to seven-septate.

Ascigerous stage in *Nectria*. Perithecia quite small, red, usually in groups on bark invaded by sphaeriaceous fungi; ascospores hyaline, one-septate, usually tan-colored in mass. (Not to be confused with another *Nectria* species which has similar perithecia, but an imperfect stage not in *Fusarium*, possibly in *Tubercularia*.)

Voronikhin's *F. albizziae* probably is a member of this species, and possibly his *Nectria albizziae* is also.

Fusarium lateritium Nees ex Fr. (*Gibberella lateritia* Snyder and Hansen)

Bright-pink to orange sporodochia, protruding from lenticels, consisting of macroconidia. In culture: pink, flesh, or orange sporodochia; mycelium white, pink, or variously colored, sometimes carmine red or white where in contact with the agar, colonies usually slower growing than those of *F. oxysporum* or *F. solani*, but faster growing than those of *F. episphaeria*. Microconidia and chlamydospores usually lacking. Macroconidia resembling those in *F. oxysporum* but usually longer, often with a tendency for the terminal cell to be hooked; three- to five-septate.

Ascigerous stage suspected on mimosa but not observed. Perithecia blue black, in groups; ascospores hyaline, curved, mostly three-septate, usually tan-colored in mass.

Fusarium roseum Lk. ex Fr. (*Gibberella rosea* Snyder and Hansen)

Bright-pink to flesh-colored sporodochia, often small but otherwise resembling those of *F. lateritium*. In culture: mycelium white, yellow, or red, very fast growing but often slow to produce conidia. Microconidia usually lacking; chlamydospores present or absent; macroconidia in flesh- to tan-colored sporodochia, apical cell not hooked.

Ascigerous stage not observed on mimosa.　Perithecia blue black; ascospores hyaline, curved, usually three-septate, tan-colored in mass.

Tubercularia vulgaris Tode ex Fr. (*Nectria cinnabarina* Tode ex Fr.)

Conspicuous pink sporodochial cushions, protruding from lenticels, composed of microconidia.　In culture: moderately scant white mycelium, producing pink to flesh-colored sporodochia of the *Tubercularia* stage in outdoor culture.

Ascigerous stage grouped around the sporodochia.　Perithecia red, resembling those of *Hypomyces solani*; ascospores hyaline, one-septate.　Observed at Washington, D. C., by Fowler and Stevenson (*3*).

Thyronectria austro-americana (Speg.) Seeler

Perithecia in clusters, light to dark brown; ascospores hyaline to pale straw-colored, three- to six-septate, muriform, often budding in the ascus and producing numerous ascoconidia.

Conidia of the *Gyrostroma* stage produced in pycnidia in the stroma, hyaline, nonseptate, orange-colored in mass.

Eutypa heteracantha Sacc.

Black perithecia grouped in a stroma, beaks protruding; ascospores hyaline, nonseptate.　Fruiting commonly on dead bark of mimosa and frequently providing the substrate upon which *Nectria episphaeria* and *Nectria* sp. were found to develop.

THE SUMAC WILT FUSARIUM

During the summer of 1946 wilting and dying of staghorn sumac (*Rhus typhina* L.) were observed by G. H. Hepting along the Blue Ridge Parkway, near Waynesboro, Va.　Examination of diseased plants disclosed that the disease was a vascular wilt, and cultures from the discolored xylem yielded a form of *Fusarium oxysporum* [6] which has been shown to be pathogenic (*20*).　Observations during the 1947 season were concerned principally with the extent of the infection in Virginia, inoculation tests, and taxonomic studies.

TAXONOMY AND NOMENCLATURE

Isolations of the causal *Fusarium* were made by tissue platings from the xylem of root, crown, trunk, branch, petiole, and base of the fruiting cluster of wilted plants.　In each case, single-spore transfers were made directly from the sporulation on the bits of tissue.　Also, numerous single-spore isolations were made directly from sporodochia found abundantly on the trunks and sometimes on the lower branches of wilted trees, especially where the sumac was located in moist or densely vegetated stands.　In repeated isolations identical clones of a *Fusarium* were obtained from the sporodochia on the bark and the discolored xylem beneath it.　These observations, together with inoculation data (*20*), showed that this wilt *Fusarium* can produce sporodochia on the bark, as in the case of the mimosa wilt fungus. *F. solani* was also isolated from the bark of diseased sumac, and its ascigerous stage, *Hypomyces solani*, was observed and identified with it culturally.

The sumac wilt *Fusarium* was typical of *F. oxysporum* in that, on potato-dextrose agar, it produced a white mycelial colony which later developed typical sporodochia.　Microconidia developed abundantly and in false heads, intercalary and terminal chlamydospores were common in the mycelium and in old conidia, and the macroconidia were characteristic of the species (fig. 2).

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[6] Original isolations were made by R. W. Davidson, of the Division of Forest Pathology, United States Department of Agriculture, and original determination as *F. oxysporum* was made by H. N. Hansen and W. C. Snyder, of the Division of Plant Pathology, University of California.
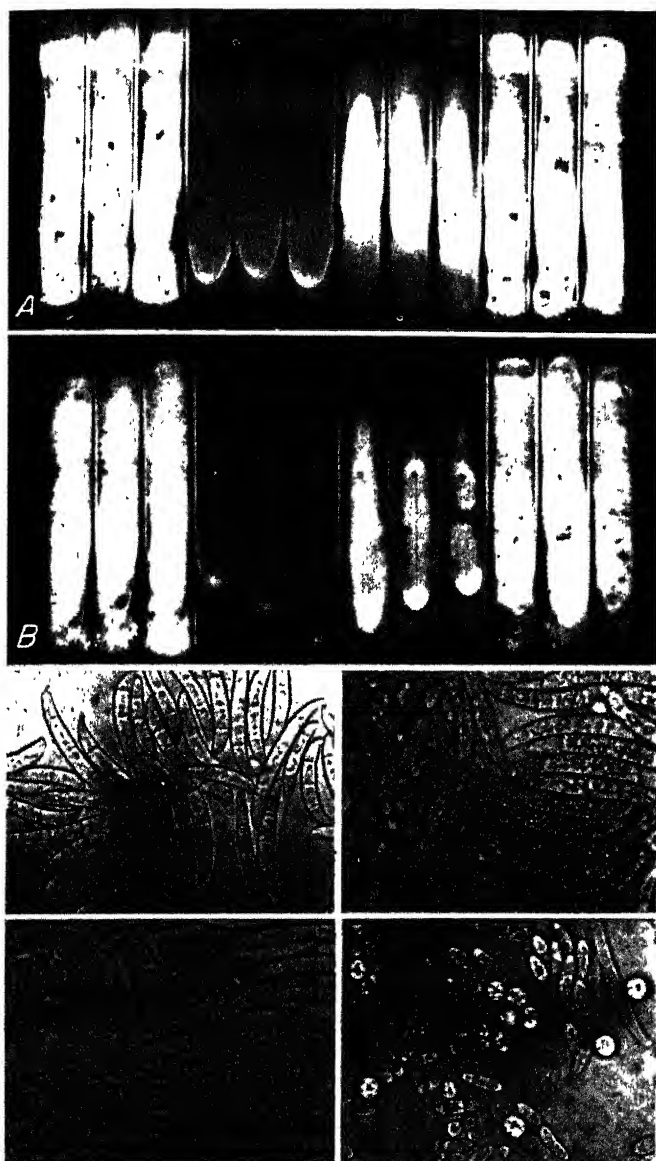
FIGURE 2.—*A*, Triplicate cultures of representative clones of *Fusarium oxysporum* f. *rhois* (first and last three tubes, natural clones; third three, going into the pionnotal clone; and second three, pionnotal mutants). *B*, Clones of *F. oxysporum* f. *perniciosum* arranged below those in *A* to show their similarity to clones of *F. oxysporum* f. *rhois*, when grown from single spores sown the same day on the same batch of medium. *C–F*, *F. oxysporum* f. *rhois: C*, Small macroconidia produced indoors; *D*, large macroconidia and microconidia of the clone shown in *C* but produced outdoors; *E*, average-sized macroconidia; *F*, microconidia and chlamydospores mixed with a few macrospores. *C–F*, × 500.

Single-spore clones of the sumac fungus have been found to belong to the species *F. oxysporum*, and these proved to be pathogenic to sumac. Since cross-inoculations with *F. oxysporum* f. *perniciosum*, the only other known vascular *Fusarium* pathogen of a tree, have been negative, the sumac pathogen is described here in conformity with the classification and nomenclature of Snyder and Hansen (*13*) as a new forma of this species. The following trinomial is proposed:

Fusarium oxysporum Schlecht. emend. Snyder and Hansen forma *rhois* Snyder and Hepting form. nov.
    Parasitic in the vascular system and causing a wilt disease of *Rhus typhina* in the Blue Ridge Mountains of Virginia.[7]

VARIABILITY IN FUSARIUM OXYSPORUM F. RHOIS

The occurrence in nature of only one clone of this vascular pathogen is in sharp contrast to the mimosa wilt fungus, for which numerous natural clones have been established. The reason for this difference may be the fact that the sumac disease so far is known in only one small area, a few miles long. It seems possible that only one natural clone has established itself or has originated in this area, whichever the case may be. The mimosa wilt, on the other hand, is widespread throughout many States in the Southeast (*19*) and in a great variety of soil and climatic environments. Such a situation would be expected to result in time in the occurrence of geographical or ecological natural clones best suited to these different environments.

Although the isolates from nature so far have belonged to one clone, this clone, when cultivated in the laboratory in pure culture on potato-dextrose agar, has proved to be very unstable. Single-spore cultures of the natural clone, which is the typical sporodochial clone of *F. oxysporum*, have repeatedly yielded several mutants each succeeding time that they were single-spored. The most common mutant which continually arises from the sporodochial natural clone is the pionnotal one (fig. 2, *A*, second three tubes), and this may be considered as an expression of the Hansen dual phenomenon (*4*).

SIMILARITY BETWEEN THE SUMAC AND MIMOSA PATHOGENS

Although *F. oxysporum* f. *perniciosum* and *F. oxysporum* f. *rhois* are distinct biologically in that neither is pathogenic to the host of the other, their similarity in culture is very close. The natural clone of the sumac fungus may be matched in appearance closely with certain natural clones of the mimosa fungus. Furthermore, some of the mutants obtained from the mimosa fungus are almost identical in appearance with those from the sumac *Fusarium* (fig. 2, *A* and *B*), and no way has been found to tell them apart in their microscopic characters. The natural clone corresponds in appearance to clone *B* of *F. oxysporum* f. *perniciosum*, and it is known to throw mutants that agree with clones *C* and *D* of that fungus. These observations

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[7] The writers are indebted to E. K. Cash, Division of Mycology and Disease Survey, Bureau of Plant Industry, Soils, and Agricultural Engineering, for her suggestion on the derivation of the forma name from *Rhus*.

further emphasize the basis for grouping them in the same species, yet keeping them separate on the basis of biological habit.

THE PINE PITCH CANKER FUSARIUM

The identity and characteristics of the *Fusarium* that causes the pitch canker disease of pine (*8*) are of particular interest (1) because this is the first disease of pines, other than seedling diseases, that has been ascribed to a *Fusarium* and (2) because of the value that this organism may have in prolonging gum flow from chipped pines in the naval stores industry (*6*). Whether the primary consideration may be the control of the pathogen responsible for pitch canker or the cultivation of, distribution of, and inoculation with a *Fusarium* beneficial in the production of turpentine and rosin, either the control or utilization of this organism depends upon an understanding of its identity and its behavior both in nature and in laboratory culture.

NATURAL DISTRIBUTION

Up to the present time the pitch canker fungus has been isolated from several species of southern pines in parts of Virginia, North Carolina, Tennessee, South Carolina, Georgia, Alabama, and Florida. Many natural cankers have been examined, especially in the North Carolina range of the disease, without finding fruiting of the *Fusarium*. This lack of fruiting of any kind has increased the difficulties in determining the kind of *Fusarium* involved. In old, inactive cankers the fungus is usually dead, and there is little promise of finding the fruiting stages on such material.

The only sporulation seen in the field on pines has been where the bark-covered wood of an active canker was artificially exposed or on the chipped surfaces of pines tapped for gum and artificially inoculated with the fungus. In these cases the *Fusarium* produces a light, white, patchy growth of mycelium on the wood itself. Later these patches often become pink and produce moderate amounts of conidia, most of which are microconidia but a few of which are macroconidia. This behavior, together with the fact that normal sporulation on undisturbed natural cankers has not been seen, suggests that the pine is not the only host of the *Fusarium*, but that under certain conditions the fungus has been able to establish itself on pine from inoculum perhaps produced on a very different host. Natural infection and distribution may take place through the aid of insects often found in connection with small new infections and observed to emerge from old cankers.

THE PINE FUSARIUM IN CULTURE

Since natural sporulation has been wanting, isolation of the pitch canker pathogen has depended upon the culture of bits of tissue taken from the wood and inner bark of active cankers (*8*). Single-spore isolations made directly from such tissue platings have given pure cultures of several natural clones.

During the 1947 season about 60 single-spore isolates of the *Fusarium* from pitch cankers in the aforementioned States and from 4 species of pine (*Pinus virginiana* Mill., *P. echinata* Mill., *P. caribaea* Mor., and *P. palustris* Mill.) were assembled. Most of these isolates
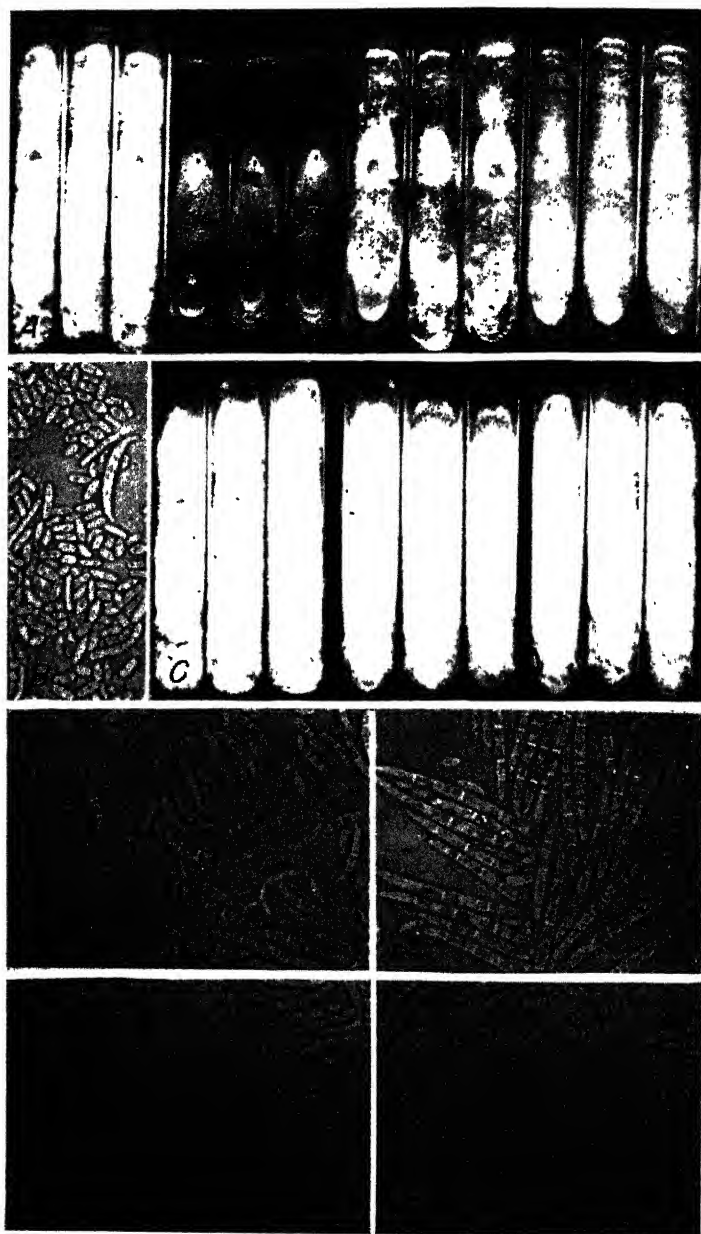
FIGURE 3.—*Fusarium lateritium* f. *pini*. *A*, Triplicate cultures of representative clones. *B*, Microconidia mixed with a few macrospores. *C*, Triplicate cultures of identical natural clones (type C) from *Pinus virginiana*, *P. palustris*, and *P. caribaea*, respectively. *D–G*, Macroconidia of different clones, all grown during the same period from single-spore cultures, showing great variation in size and shape. *B* and *D–G*, × 500.

represent natural clones, but a few were laboratory mutants.[8] These cultures provided the basis for the observations made on taxonomy and pathogenicity.

· All natural clones in culture in the laboratory were characterized by a white fluffy mycelium which, in some clones, tended with age, to become pigmented in shades of blue, gray blue, green blue, and purple, especially in diffuse daylight. Many clones developed a purple pigment in the mycelium lying in contact with the potato-dextrose-agar slant. Some clones produced dark-bluish sclerotia. Many other natural clones differed in these and in other characters such as colony texture, configuration, zonation, and rate of growth (fig. 3, *A*).

Microscopically the laboratory-grown cultures were unsuited for taxonomic study, because macroconidia were almost entirely lacking even though the cultures were grown in light. In this absence of macroconidia the pine *Fusarium* could have fallen into the species *F. moniliforme*, into *F. oxysporum* if certain swellings in the mycelium were interpreted as chlamydospores, or into *F. lateritium*.

A good supply of normal macroconidia in sporodochia were produced on potato-dextrose cultures kept outdoors in the fall and subject to wide fluctuations in diffuse light and in temperature. Companion cultures of the same clones made at the same time on the same batch of medium but incubated in the laboratory near the window, produced no sporodochia.

The sporodochia of all natural clones grown outdoors were orange-colored, and the masses of macroconidia were often in sufficient abundance to give the entire slant an orange cast. The masses of macroconidia began to appear about a month after the cultures were placed outdoors, but they reached their greatest development in about 6 weeks.

Macroconidia were three- to five-septate, with a tendency toward the formation of a slight hook of the apical cell, characteristic of the small-spored types in *F. lateritium*. The apical-cell hook, the preponderance of macroconidia in optimum culture, and the absence of both chlamydospores and chain-formed microconidia place the fungus in *F. lateritium*. The fungus was first published under this name in 1947 (7).

The variation in cultural forms and in spore characteristics is evident in figure 3. Mutations from natural clones were occasionally obtained in the laboratory through single-spore transfer from single-spore cultures. The commonest mutant, recovered from several clones, was a pionnotal variant. Such cultures produced little aerial mycelium and a slime of spores on the surface of the agar and usually were deeper purple than the parent. The macroconidia of the pionnotal clones were generally much longer than those of the parent (fig. 3). Other mutants differed in the amount of pigmentation, amount and character of mycelium, and rate of growth.

[8] Cultures and fresh canker material from Georgia and Florida were supplied by R. P. True and from Alabama by R. M. Lindgren, both of the Division of Forest Pathology.

TAXONOMY OF THE PINE FUSARIUM

After a study of cultures from sporodochial natural clones had established the fact that the pine *Fusarium* was *F. lateritium*, it was necessary to determine whether only clones of this species from pine would cause pitch canker and gum flow. To test this point, fresh isolates of a *F. lateritium* from *Albizzia* were used to make parallel inoculations in Virginia pine in conjunction with isolates of the pine form. Cankers and gum flow were obtained with isolates from pine only.

In view of the distinctive pathogenicity on pine of the isolates obtained from pitch cankers and the unique economic potentialities of this fungus in the naval stores industry, it is proposed to designate the pine *Fusarium* as a new form as follows:

F. lateritium Nees emend. Snyder and Hansen forma pini Hepting form. nov.

Pathogenic on the trunk, leader, and branches of pine (*Pinus virginiana, P. echinata, P. caribaea,* and *P. palustris*), causing thereon cankers characterized by heavy gum flow. Also prolongs gum flow from these hosts when applied to fresh wounds on them. Natural habitat, southeastern United States.

Clones grown on potato-dextrose agar in diffuse daylight include the following and various intermediate stages between them:

Clone A.—Common clone (fig. 3, *A*, first three tubes). Fast-growing, raised mycelial thallus, white through vinaceous shades, producing abundant microconidia in false heads indoors and abundant three- to five-septate macroconidia on sporodochia outdoors.

Clone B.—Sclerotial clone (fig. 3, *A*, third three tubes). Similar to *A*, but producing abundant sclerotia or blue-black plectenchyma.

Clone C.—Similar to clones *A* and *B*, but producing neither sporodochia nor sclerotia (fig. 3, *A*, fourth three tubes).

Clone D.—Pionnotal clone (fig. 3, *A*, second three tubes). No aerial mycelium. Macroconidia and microconidia in a slime over the agar. Whitish to vinaceous shades.

Clone E.—Very slow growing, bright purplish blue, producing mostly microconidia even outdoors, with semiraised mycelium.

The only other *Fusarium* isolated from pine wounds in this study was *F. roseum*. Both red and yellow-brown natural clones of this species were recovered. They failed to produce lesions when inoculated on *Pinus virginiana*. The *Gibberella rosea* stage was obtained in single-conidium cultures incubated outdoors during October and November.

DISCUSSION

In isolating clones of *Fusarium* species from nature, it is desirable that single-spore or single-hyphal-tip techniques be used either directly from the host or from the first tissue isolates. One of the most important reasons for this requirement is the tendency of all three pathogenic Fusaria herein described to live in close association with other Fusaria, notably *F. solani*, not only on the same host, but sometimes even in the same bark pustule or in the same vascular tissue. Natural clones were maintained through successive culturing by single-sporing all transfers and retaining those that were identical with the original clone.

The importance in identification of working with a large number of cultures transferred the same day on the same batch of medium was clearly shown by the varying effects of different environments on the appearance of cultures of parallel series during the 5 months in which these studies were carried on. For example, cultures of *F. lateritium* f. *pini* kept indoors in diffuse light produced abundant microconidia and practically no macroconidia, and such cultures would probably never have been identified as that species. Parallel cultures stored outdoors produced excellent sporodochia, abundant macroconidia, and almost no microconidia and were readily placed under *F. lateritium*. Cultures of *F. oxysporum* f. *perniciosum* kept indoors produced for the most part relatively short macroconidia, although some produced long macroconidia; whereas outdoor cultures produced macroconidia that were of average length for *F. oxysporum* generally. Thus some special handling, approaching more natural conditions (*17*), is often required to get good development of the structures used in identification, and the parallel study of many isolates is necessary to acquaint an investigator with the variations that may occur in a given species and forma.

The pine canker and mimosa wilt fungi illustrate in a striking way the multiplicity of morphologic clones of certain Fusaria in nature. These results, together with those of Borlaug (*1*) on flax wilt, Nelson, Coons, and Cochran (*11*) on celery wilt, and Snyder (*12*) on pea wilt, fail to substantiate Miller's (*10*) concept that usually there is only one "wild type," or natural clone, for a given *Fusarium*. The work just cited and the current studies on *F. oxysporum* f. *perniciosum* and *F. lateritium* f. *pini* show for several Fusaria wide variability in isolates made on the same medium directly from nature. Comparisons made by some workers of isolates of a given *Fusarium* obtained from a variety of sources over a wide range of years are not accepted by Miller as valid comparisons of natural clones, since some mutations undoubtedly took place in the meantime. In the case of Miller's muskmelon *Fusarium* in Canada (*9*) and of the sumac *Fusarium*, the occurrence of one predominating natural clone seems to be the rule. However, the variation in natural clones of the flax wilt, pea wilt, celery wilt, pine canker, and mimosa wilt Fusaria, in addition to the experience of the writers with other Fusaria, indicates that Miller's assertion (*10*) that one "wild type" predominates and that variation in types isolated directly from nature "must be relatively infrequent" should not be applied to Fusaria in general.

The morphological variability shown within the pathogenic formae of the mimosa wilt and pine canker Fusaria follows the pattern of other Fusaria (*1, 9, 10, 11, 12, 13, 14, 16, 22*) and poses important questions concerning the identification of such pathogens, procedures for the maintenance of stock cultures, selection of the clone for deposition in type-culture collections, and choice of clones for use in projects aimed at nomenclature, disease resistance, and control. Certainly an intelligent approach to the problems presented by these diseases requires recognition of the phenomenon of morphologic and physiologic variability demonstrated to occur in nature.

SUMMARY

Clones of Fusaria pathogenic to species of *Albizzia, Pinus,* and *Rhus,* isolated in pure culture from trees in numerous locations, provided the bases for the comparative studies on the cultural characteristics, taxonomy, and nomenclature of these fungi. Single-spore or single-hyphal-tip cultures made directly from the host or from fresh-tissue cultures were used throughout the work, both in the isolation of natural clones and in subsequent subculturing.

Although one or more clones of each of the three pathogens were shown to be variable in culture through mutation, two of the pathogens—those of *Albizzia* and of *Pinus*—were found to exist in nature in a highly variable state, each consisting of numerous distinct, morphologic clones.

The trinomial *Fusarium oxysporum* f. *perniciosum* (Hepting) Toole is accepted for the mimosa wilt pathogen; *F. oxysporum* f. *rhois* Snyder and Hepting is proposed for the sumac wilt pathogen; and *F. lateritium* f. *pini* Hepting is proposed for the pine pitch canker *Fusarium.*

Procedures applicable to pure-culture isolation, the maintenance of original clones, and the induction of optimum sporulation are outlined for each pathogen, and brief descriptions of clones are provided as a guide to their identification. Sporodochia of the pitch canker *Fusarium* were obtained only in outdoor culture under conditions of fluctuating light and temperature.

Consideration is given also to the saprophytes commonly associated with the above-named pathogens in diseased trees.

Some sporodochia on the bark of diseased mimosa and sumac yielded pure cultures of the pathogens, whereas others proved to be those of saprophytic Fusaria. No sporulation of the pitch canker *Fusarium* was observed on natural cankers in the field, but some occurred on chipped faces of pines artificially inoculated.

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PROBLEMS RELATING TO THE REMOVAL OF DDT SPRAY RESIDUE FROM APPLES[1]

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I. EFFECT OF WASHING ON REMOVAL OF DDT RESIDUE

The increased use from 1931 to 1946 of acid lead arsenate-petroleum oil mixtures, of cryolite-petroleum oil mixtures, or both for the control of the codling moth in the orchards of north-central Washington necessitated the development and adoption of uniform chemical washing practices for the removal of spray residues. Generally, a lead arsenate-oil or cryolite-oil spray program consisted of five to eight cover sprays, at least three of which contained oil at the rate of 2 quarts or more per 100 gallons of spray. To remove the spray residue from fruit sprayed with these mixtures, it has been necessary to use a two-unit (tandem) type washer.[2][3] Most of the commercial warehouses in the north-central Washington fruit district have employed a fruit washing system similar to the one outlined below.

First unit—40 to 60 pounds of sodium silicate per 100 gallons of water at 90° to 100° F.

Warm water rinse.

Second unit—1.5 percent hydrochloric acid at 90° to 100° F.

Copious fresh water rinse.

The results obtained in codling moth control from the use of DDT in the experimental spray plots conducted by various investigators of State and Federal agencies forecast the widespread use of this material in commercial orchards in 1947. Experimental washing studies were therefore undertaken to determine the effectiveness of commercial warehouse washing practices when employed for the removal of DDT from apples.[4][5]

Fruit from experimental plots were selected for residue sampling. All samples were taken by sampling at random two or three fruits from each picked box until one-half to one full box was collected. All samples were divided into eight subsamples and two of the subsamples were analyzed for original DDT residue. The remaining samples were washed in a single-unit Cutler washer with the following characteristics:

Time of one revolution_____ 3.3 seconds
Length of step-over (carrier)_____ 4 inches
Length of tank_____ 33 inches
Time in machine_____ 26.8 seconds

Two samples were washed in 1.5 percent hydrochloric acid at 100° F.; two samples were washed in 60 pounds of sodium silicate per 100 gallons of water at 100°; and then, in an effort to duplicate the tandem wash used in commercial practices, two samples were washed in the sodium silicate at 100° and rerun through the same machine after it had been cleaned and refilled with 1.5 percent hydrochloric acid at 100°.

DDT residue was determined by treating approximately 10 fruits in the laboratory with a known volume of toluene in a tumbling machine for a 5-minute period. An aliquot of the toluene was treated with 50 milliliters of liquid ammonia and enough metallic sodium to form a permanent blue color in the ammonia layer. After standing one-half hour, the excess sodium was destroyed with methyl alcohol and the samples evaporated to dryness on a hot plate. The sample was picked up with 25 milliliters of water, 5 milliliters of nitric acid was added, and the chlorine was determined by the Volhard method.

Forty-two samples with an original residue load ranging from 5.0 to 22.3 parts per million were used in each of the washing trials. Replicate samples were used in determining the original residue load and in each of the washing trials.

The percentage of samples with a DDT residue equal to or greater than the present permissible tolerance of 7 parts per million and the percentage of samples showing reduced amounts of residue due to washing are presented in table 1. Slightly less than one-half (47.6 percent) of the samples that received no treatment carried a DDT residue of more than 7 parts per million. The hydrochloric acid treatment decreased the percentage of samples with a DDT residue of more than 7 parts per million to 35.7 percent, or an increase of 25 percent over the samples that received no treatment. The sodium silicate treatment and the tandem treatment decreased the percentage of samples with a DDT residue of more than 7 parts per million to 28.6 percent, or an increase of 39.9 percent over the samples that received no treatment. For DDT residue removal, the one-bath sodium silicate treatment was more efficient than the one-bath hydrochloric acid treatment and as efficient as the tandem treatment.

Significant amounts (at the 5-percent level) of DDT residue were removed from 78.6 percent of the hydrochloric acid washed samples, 88.1 percent of the sodium silicate washed samples, and 90.5 percent of the tandem washed samples. Although the washing treatments removed significant amounts of residue, none of them removed sufficient amounts of DDT from all samples to make the methods practicable for commercial use.

TABLE 1.—*Percentage of samples showing DDT residue above 7 p. p. m. after washing and percentage of samples showing reduced amounts of residue*

Treatment	Samples above 7 p. p. m.	Effectiveness of treatment
	Percent	*Percent*
No washing	47.6	0
Hydrochloric acid	35.7	25.0
Sodium silicate	28.6	39.9
Tandem	28.6	39.9

For the purpose of comparison, the samples were divided into three classes: (1) Those samples with an original DDT residue of less than 7 parts per million; (2) those samples with an original DDT residue of more than 7 parts per million; and (3) all samples regardless of the amount of the original DDT residue. The mean amount of DDT residue removed by the treatments in each of the above classes in presented in table 2.

TABLE 2.—*Mean amount of DDT residue removed*

Treatment	Class 1 Original residue less than 7 p. p. m.	Class 2 Original residue more than 7 p. p. m.	Class 3 All samples
	P. p. m.	*P. p. m.*	*P. p. m.*
Hydrochloric acid	1.5	1.7	1.6
Sodium silicate	1.9	3.1**	2.5**
Tandem	2.2	3.3**	2.7**

**Significant at the 5-percent level.

The sodium silicate wash and the tandem wash removed significantly greater amounts of DDT residue in class 2 and class 3 samples than the hydrochloric acid wash. In the class 1 samples, all treatments removed approximately the same amount of DDT residue. In all three classes, the amount of DDT residue removed by the sodium silicate wash was very nearly the same as that removed by the tandem wash, and there was no significant difference between these two treatments. The sodium silicate wash was more efficient in residue removal than the hydrochloric acid wash and was as efficient as the tandem wash.

Regression analysis comparing the original DDT residue present on the unwashed samples and the amount removed by washing was made of each treatment and no significant relationship was found. Percentage residue removal was calculated for each sample, for each treatment, and was found to vary from a negative 32.8 percent to a positive 69.5 percent. No correlation could be established between the percentage of DDT residue removed and the amount originally present. The average removal by the hydrochloric acid treatment was found to be 21.3 percent, by the sodium silicate treatment, 29.5 percent, and by the tandem wash treatment, 35.6 percent.

II. EFFECT OF CERTAIN STICKERS AND SPREADERS ON DDT RESI-DUE AND OF OVERHEAD SPRINKLING ON REMOVAL OF DDT RESIDUE

During the 1947 growing season in north-central Washington there was an increased use of orchard sprinkling irrigation systems and, as expected, a change of spray programs from lead arsenate-oil mixtures and cryolite-oil mixtures to DDT dispersed in water. Studies were accordingly made to determine the effect of certain stickers and spreaders on the DDT residue and the effect of overhead sprinklers on the removal of DDT coverage from fruit trees. The water from under tree sprinklers usually does not wet the fruit on the lower limbs until late in the season when the weight of the fruit bends it into the sprinkler's stream. For this reason, the effect of under-tree sprinklers was not a problem to be investigated at this time. The following work with certain stickers and spreaders and overhead sprinklers was undertaken at the Tree Fruit Experiment Station in Wenatchee, Wash., during the 1947 growing season.

Four adjoining Starking Delicious apple trees, approximately 12 years of age, were selected in order that one overhead sprinkler set up in the center could adequately cover all the trees. An oscillating sprinkler was used with a one-eighth-inch nozzle at 7° above the horizontal. The sprinkler was operated at 22 pounds' pressure, and delivered approximately 5 acre-inches of water during the 24-hour period.

Samples of fruit were taken at random over the entire tree before sprinkling and after 24 hours of continuous sprinkling. The trees were allowed to dry after sprinkling, before the samples were taken. DDT residue was determined by the method given in part I of this paper. Four different spray programs were used. All sprays were applied by one man using a 100-gallon Hardie portable sprayer with 425 pounds' pressure at the pump. The results are shown in table 3.

TABLE 3.—*DDT deposits before and after irrigation by sprinkling for 24 hours*

Plot No.	Spray program [1]	Mean DDT deposit—		Mean difference
		Before sprinkling	After sprinkling	
		P. p. m	*P. p. m*	*P. p m.*
1	2 lbs. 50-percent DDT per 100 gals of water	13.9	13.1	0.8
2	2 lbs. 50-percent DDT plus 2 qts. light-grade summer petroleum oil per 100 gals. of water	18.8	19.9	−1.1
3	2 lbs. 50-percent DDT plus ⅙ lb. dry-casein-type spreader per 100 gals. of water	15.9	14.0	1.9
4	2 lbs. 50-percent DDT plus ¼ pt. liquid-soap-type spreader per 100 gals. of water	9.1	10.1	−1.0

[1] There were 8 replicates of each treatment.

The addition of 2 quarts of light-grade summer petroleum oil to the spray program (plot 2) gave a significant increase in DDT residue over the spray program on plot 1. The addition of one-sixth pound of dry-casein-type spreader (plot 3) did not give a significant increase or decrease in DDT residue over the spray program on plot 1. The addition of one-fourth pint of liquid-soap-type spreader (plot 4) gave a significant decrease in DDT residue over the spray program on plot 1.

The study of the effect of overhead irrigation sprinklers on the removal of DDT indicated that 24 hours of overhead sprinkling did not reduce the amount of DDT adhering to the fruit. In no case was there a significant difference in DDT residue before and after sprinkling.

SUMMARY

The results of two studies relating to DDT spray residue on apples are reported herein. The first relates to the effect of washing on the removal of DDT residue, the second to the effect of certain stickers and spreaders on DDT residue and of overhead sprinkling on removal of DDT residue.

The results of the first study may be summarized as follows:

When 42 apple samples with an original DDT residue ranging from 5.0 to 22.3 parts per million were washed by three different treatments, a high percentage of the washed samples had a significant amount of DDT residue removed. Nevertheless, none of the washing treatments removed sufficient residue from all samples to make these treatments practicable for commercial use. Sixty pounds of sodium silicate per 100 gallons of water at 100° F., in a single-unit washer gave a more efficient DDT residue removal than 1.5 percent hydrochloric acid at 100° F., in a single-unit washer, and was equally as effective as the tandem wash of sodium silicate followed by hydrochloric acid.

When the original DDT residue on apples exceeded 10 parts per million, the standard washing practices in north-central Washington were not sufficient to consistently reduce this residue to a point equal to or less than the permissible tolerance limit of 7 parts per million.

In the second study it was found that—

(1) The addition of light-grade petroleum oil significantly increased the DDT residue over the straight DDT spray.

(2) The addition of dry-casein-type spreader did not significantly increase the DDT residue over the straight DDT spray.

(3) The addition of liquid-soap-type spreader significantly decreased the DDT residue over the straight DDT spray.

(4) Twenty-four hours of overhead sprinkling irrigation with an oscillating sprinkler that delivers approximately 5 acre-inches of water in a 24-hour period did not remove significant amounts of DDT residue from fruit sprayed with wettable DDT, wettable DDT and petroleum oil, wettable DDT and dry-casein-type spreader, or DDT and liquid-soap-type spreader.

COMPOSITION AND DIGESTIBLE ENERGY OF HAYS FED TO CATTLE[1]

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INTRODUCTION

The evaluation of feeds is the subject of a report by a committee of the American Society of Animal Production written by Mitchell (*10*).[2] This reviewed the relations that have been found to exist between constituents determined chemically and certain items in the utilization of feeds. The close negative relation between crude fiber content and the digestibility of the organic matter of feeds by different species of animals was emphasized especially. The committee suggested that further studies along these lines might yield valuable results.

Dissatisfaction with the standard methods of analysis has been expressed frequently. Crampton and Forshaw (*4*) among others, have attempted to use more detailed determinations, especially of the carbohydrate fractions, in·studies of the relation of composition to the usefulness of feeds. The results have been disappointing. So far no carbohydrates or grouping of carbohydrates known or supposed to be utilizable have been found which are clearly related to the energy animals can obtain from feeds.

In the present limited study it was hoped that some definite relations between composition and useful energy might be found if the number of variables were reduced as far as possible. For this purpose investigators known to have conducted experiments on the energy metabolism of farm animals were asked if they could supply samples of essentially pure stands of grasses or hays that had been used as the sole ration in energy utilization studies with cattle. To supplement the data supplied by the cooperating investigators, these samples were to be analyzed in this laboratory as completely as practicable, with especial attention to the carbohydrate fractions. The items of composition were then to be compared with the results of the digestion experiments.

MATERIAL AND METHODS

The conditions proposed above limited the number of samples that could be found. Eventually 25 were received, as follows: 10 of

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timothy, of which No. 9 was grass fed fresh; 1 of bromegrass; 1 of oat hay; 1 of Sudan grass; 1 of mixed grass, largely redtop, fed fresh (No. 8); 7 of alfalfa; 1 of red clover; 1 of alsike; 1 of soybean hay; 1 of lespedeza. With the 2 exceptions noted, all were fed as hay.[3]

Accompanying each sample were the results that had been obtained for digestible energy and the digestibility of the dry matter. In many cases the metabolizable energy had been determined either directly or by calculation. The results of the standard feeding-stuff analyses also were provided with many of the samples.

The samples were analyzed in the writers' laboratory by the methods used in a previous study of timothy (*11*) except that the extraction was modified for dry samples. These were extracted in a Soxhlet apparatus, first with anhydrous ethyl ether, then with 95 percent ethyl alcohol. The material extracted by alcohol did not all remain in solution but dissolved when enough water was added to make the alcohol concentration 80 percent.

The items or groups used in the comparisons were (1) protein, (2) lignin, (3) cellulose, (4) hemicellulose, (5) total carbohydrate as suggested by Crampton and Whiting (*6*) including hemicellulose, starch if present, fructosans if present, and the sugars, all calculated to the starch equivalent, and (6) crude fiber.

These categories of materials were compared with (1) the digestibility of the energy, (2) the digestible energy in calories per gram, and (3) the digestibility of the dry matter. Since the heats of combustion of the samples differed only slightly the relations of constituents to (1) and (2) are so nearly identical that only the former are presented. The relations to (1) and (3) also are quite similar. These relationships were graphed and the corresponding correlation coefficients and regression equations were calculated.

RESULTS OF ANALYSES

It was found that metabolizable energy had not been determined for 7 of the samples and that the data for calculating it were not available. Therefore this item could not be used in the comparisons. The relation between metabolizable energy and digestible energy for the remaining 18 samples was plotted and the correlation coefficient was calculated. The graph was a straight line one with $r = +0.948$. In this limited group of samples, then, the relation between these items is very close.

The data used in the rest of the calculations are presented in table 1. The figures for digestibility and many of those for crude fiber were supplied by the cooperating investigators. The remainder of the items were determined in this laboratory.

TABLE 1.—*Digestibility and composition of hays*

Sample No.	Species	Digest-ible energy	Digestibility of—		Composition (percent of dry weight) of—					
			Energy	Dry matter	Pro-tein	Hemi-cellu-lose	Total carbo-hy-drates	Cellu-lose	Lignin	Crude fiber
		Cal. per gm.	*Percent*	*Percent*						
7	Timothy_____	2,567	57.7	60.4	5.98	14.83	28 95	30.14	9.85	33.0
9	____do_____	3,534	78 6	80.8	11.36	13.28	25 51	24.94	6.92	24.0
12	____do_____	2,493	55 7	56.7	5.41	17.10	27.28	33.72	10.56	34.8
15	____do_____	2,498	56.4	59.3	8.31	15.03	19.91	32.62	10.51	33.5
16	____do_____	2,856	62.4	64.4	8.00	17.61	22 01	37.20	9.60	34.7
17	____do_____	2,387	53.2	54 8	5.89	15.57	25.98	34.53	11.49	34 1
19	____do_____	2,232	49.6	47.3	7.27	16.34	22.81	34.20	11.53	34.9
22	____do_____	2,785	62.0	65.3	9.07	17.20	23.39	30.54	9.69	33.1
24	____do_____	2,569	59.6	61.7	9.44	13.51	23 82	28.13	9.82	30.0
25	____do_____	2,828	64.6	67.2	8.78	16.07	23.77	27.12	8 16	29 0
1	Bromegrass_____	2,949	67 6	69.4	12.15	15 97	20.85	30.98	7.58	31.1
6	Oat hay_____	3,033	66.8	67.5	13.75	15.61	18.95	30 52	8.54	30.0
8	Mixed_____	2,332	51.7	62.5	7.28	14.89	25 54	31.29	9.45	32.8
23	Sudan_____	2,861	67.8	_____	12.59	13.62	22.22	27.90	8.24	27.2
2	Alfalfa_____	2,670	59 7	60.4	16.73	8.84	13.90	27.09	11.14	33.4
3	____do_____	3,032	67.0	67.6	22.00	8.26	13 52	23 51	8.97	26 1
10	____do_____	2,809	61.2	62.2	18.47	9.60	11.80	25.50	10.74	30.9
11	____do_____	2,463	54.5	56 2	14.43	9.76	12.35	32 60	11.92	32.8
14	____do_____	2,508	56.7	59.0	16 26	10 63	12.48	28.66	11.12	32.8
18	____do_____	2,659	58.1	59.2	17.84	9.34	11.65	26.88	11.01	30.6
21	____do_____	2,466	54.7	56 6	13.78	9.52	13.08	34.00	12.91	40.5
4	Red clover_____	2,599	60.1	59.9	12.50	8.75	16.01	28.77	11.00	30.4
5	Alsike_____	2,522	57.0	58.8	11.24	12.58	15.80	31.61	11.43	30.2
20	Soybean hay_____	2,889	63.7	60 7	11.24	13.15	19.54	35.20	11.38	33.1
26	Lespedeza_____	2,596	57.5	58.0	12 15	9.29	16.01	27.19	13.19	32.2

The relation between the protein content of the samples of timothy and alfalfa and the digestibility of their energy is shown in figure 1.
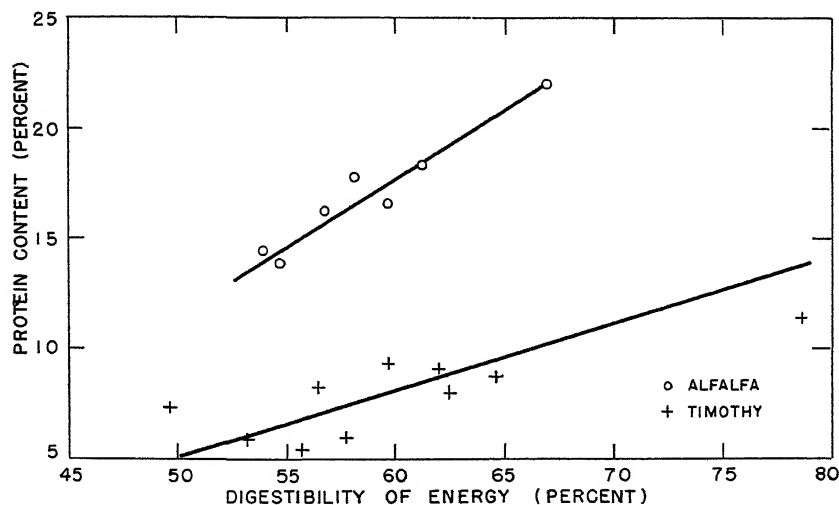
FIGURE 1.—Relation between protein content of the samples of timothy and alfalfa and the digestibility of their energy. For alfalfa $r = +0.966$, for timothy $r = +0.786$, for both $r = +0.244$.

In each of the species this relation is highly significant, but at different levels of protein content. When all 17 samples are considered the relation is not significant. Similar specific differences were evident to a greater or less extent in all the relations studied.

It was considered advisable then to study the 10 timothy samples and the 7 alfalfa samples separately and to ignore the other species. This reduced the number of samples in each comparison greatly and so decreased the value of the results.

Such relations among the major items considered as were found to be significant are listed in table 2. No significant relations were found between digestibility and content of hemicellulose or of total carbohydrates.

For the timothy and alfalfa samples, separately, the regression equations for calculating the digestibility of the energy and the digestibility of the dry matter, respectively, from the content of lignin, of crude fiber, and of protein are presented in table 3. Also included are the direct correlation coefficients, the standard deviations of the two digestibilities, and the standard error of estimate of each of these when calculated from the constituent indicated.

The large "a" values in some of these equations show the danger of extrapolating beyond the range of the data at hand. They indicate either that the relations would not follow a straight line at extreme values of the variables, or that a wider range of data would result in an appreciable change in the slope of the regression line.

TABLE 2.—*Correlation coefficients*

Variables	Percent lignin	Percent crude fiber	Percent protein	Percent cellulose
Timothy:				
Digestibility of energy	−0.962	−0.872	+0.786	−0.689*
Digestibility of dry matter	−.954	−.842	+.761*	−.689*
Alfalfa:				
Digestibility of energy	−.932	−.787*	+.966	−.899
Digestibility of dry matter	−.939	−.784*	+.966	−.889

*Significant at the 5-percent level; all others at the 1-percent level.

TABLE 3.—*Regression equations, digestibility of constituents*

TIMOTHY

y	x	$r-ry$	Equation	Sy	$Sy.x$
	Percent				
Digestibility of energy	Lignin	−0.962	$y=113.1−5.41x$	7.94	2.29
	Crude fiber	−.872	$y=123.8−2.00x$	7.94	4.13
	Protein	+.786	$y=33.2+3.37x$	7.94	5.21
Digestibility of dry matter	Lignin	−.954	$y=120.3−5.96x$	8.84	2.83
	Crude fiber	−.842	$y=130.3−2.14x$	8.84	5.06
	Protein	+.761	$y=33.0+3.62x$	8.84	6.08

ALFALFA

Digestibility of energy	Lignin	−0.932	$y=96.5−3.39x$	4.36	1.73
	Crude fiber	−.787	$y=84.6−0.79x$	4.36	2.95
	Protein	+.966	$y=32.7+1.53x$	4.36	1.24
Digestibility of dry matter	Lignin	−.939	$y=93.9−3.04x$	3.88	1.46
	Crude fiber	−.784	$y=83.0−0.70x$	3.88	2.64
	Protein	+.966	$y=36.9+1.36x$	3.88	1.10

DISCUSSION

Crampton and Forshaw (4) compared the gains in weight of rabbits with the composition of the grass clippings used as feed. Lignin was negatively correlated with gains, but the result did not reach even the 5-percent level of significance. The very high correlation of −0.978 was found by Lancaster (8) between the lignin content of 14 feeds and the digestibility of their organic matter by sheep. The feeds included both succulents and hays. Lancaster concluded that the superiority of lignin as a factor in feed evaluation is not clearly established. The determination is not yet satisfactory and further studies are needed.

The results of the present study give further evidence of the importance of this relation. Lignin content is closely related to digestibility of energy in both timothy and alfalfa, and thus in the limited sampling available, it is shown to be an excellent basis for calculating digestibility in each of the species. It should be noted, however, that the regression coefficients, which measure the change in digestibility corresponding to an increase of 1 percent in lignin content, are strikingly different for the two species, −5.41 for timothy and −3.39 for alfalfa (table 3).

Reports on the relation of crude fiber to the digestibility of the organic matter of feeds are more numerous. Some recent results are summarized in table 4. McMeekan's figures include a wide variety

TABLE 4.—*Relation between digestibility of organic matter and crude fiber conten*

Source of data	Number of items	Correlation coefficient	Regression coefficient
Cattle.			
Axelsson (1)			−0.879
Brouwer and Dijkstra (3)	9	−0.784	−1.134
Brouwer and Dijkstra (3)[1]	10	−.557	−.515
Duckworth (7) American cattle [1]	101	−.680	−.918
Duckworth (7) Zebu cattle [1]	166	−.417	−.511
Sheep:			
Brouwer and Dijkstra (3)	38	−.674	−.992
Brouwer and Dijkstra (3)[1]	22	−.525	−.720
Brouwer and Dijkstra (3)[1]	15	−.599	−.479
Brouwer and Dijkstra (3)[1]	48	−.518	−.564
Lancaster (8)	14	−.944	[2] −1.698
McMeekan (9)[1]	50	−.944	−.960

[1] Calculated from the results of digestion experiments assembled from the literature.
[2] Calculated from Lancaster's data.

of feeds, succulents, silages, roughages, and concentrates. Lancaster's include succulents and roughages. The rest are largely limited to grasses and hays. Duckworth included only roughages exclusive of silages.

All the correlation coefficients listed in table 4 show definite trends, but only the highest ones indicate a relationship close enough to permit the constituent to be used as a reasonably satisfactory measure of digestibility.

The regression coefficients also vary widely. Axelsson (2) has explained some of the variations in this figure on the basis of differences in experimental technique and has emphasized the need for the standardization of methods in digestibility trials.

The results reported here for crude fiber (tables 2 and 3) are well within the range of those assembled from the literature (table 4). In

the samplings of both timothy and alfalfa the relation between crude fiber content and digestibility of energy is significant, though not as close as the lignin relationship. Here, again, the differences in the regression coefficients relating differences in crude fiber content to differences in digestibility are striking, −2.00 for timothy and −0.79 for alfalfa.

The regression coefficients found by other workers (table 4) also vary widely, from −0.479 to −1.698. It is quite impossible to select from these varying values one which could be used with confidence in calculating the digestible energy of feeds from their crude fiber content.

In the alfalfa samples the protein content is very closely related to the digestibility of energy, but in the timothy samples this relation barely reaches the 1-percent level of significance.

The cellulose relationships differ in the same way as those of protein but are at an appreciably lower level of significance.

For neither timothy nor alfalfa do any of the groupings of carbohydrates studied show a significant relation to the energy yield. These results confirm at least the latter part of a statement made by Crampton and Jackson (*5, p. 339*), "Protein and fiber fail as indices of nutritive value as do all other fractions reported in this paper."

The relations found in this study are limited in value because of the small number of samples included. Those with lignin, however, are extremely close, and different for the two species. It is likely that they would be much less marked in studies of mixed herbage whose botanical composition varied widely.

SUMMARY

Twenty-five samples of hays received from various laboratories in the United States were studied. These hays had served as the sole ration in feeding experiments with cattle. Each sample was from an essentially pure stand of a single species of grass or legume.

The samples were analyzed chemically and their composition was compared with the results of the feeding trials.

Comparison of the digestible and the metabolizable energy of the 18 samples for which the latter value had been determined show a very close relation between the two.

Species differences were so pronounced that it was considered advisable to make separate studies of the 10 samples of timothy and the 7 of alfalfa. The other samples, one each of 8 species, were not included. Thus the number of samples used in the comparisons was small.

The lignin, protein, cellulose, and crude fiber content of the samples all are related more or less closely to their yield of energy but at differing levels of these constituents for the two species.

When applied to the samples of each of the two species separately, the lignin content serves as an excellent means of estimating the digestibility of energy and of dry matter.

Crude fiber content also is related significantly to the digestibility of energy, but less closely than the lignin content.

The regression coefficients relating differences in a constituent to differences in digestibility differ widely for the two species.

The carbohydrate fractions studied, other than cellulose, show no significant relation to the digestibility of either energy or dry matter.

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THE ORIGIN OF BREEDS OF CHICKENS [1]

By D. C. Warren, *poultry geneticist, and* Herman Smith, *research assistant, Kansas Agricultural Experiment Station*

INTRODUCTION

In Edward Brown's [2] monumental work entitled "Poultry Breeding and Production," he quotes an article by W. B. Tegetmeier, which appeared in the Field of September 26, 1885. In this article, Tegetmeier, who was an associate of Charles Darwin, questions Darwin's theory that all breeds of poultry have their origin in the wild jungle fowl, *Gallus gallus* (*G. furrugineus*). Brown (p. 5) quotes Tegetmeier as follows:

> But it is with regard to the Eastern Asiatic type of fowl . . . that my doubts as to the descent from the *G. furrugineus* are strongest. We have in the Cochin a fowl so different from the ordinary domestic birds that when first introduced the most ridiculous legends were current respecting it. Putting these on one side, we have a bird with many structural peculiarities that could hardly have been induced by domestication. Thus, the long axis of the occipital foramen in the Cochin is perpendicular, in our old breeds horizontal, a difference that could never have been bred for, and which it is difficult to see could be correlative with any other change. The same may be said respecting the deep sulcus or groove up the center of the frontal bone. The extraordinary diminution in the size of the flight feathers and that of the pectoral muscles could hardly have been the result of human selection and careful breeding, as the value of the birds as articles of food is considerably lessened by the absence of flesh on the breast. Nor is the extreme abundance of fluffy, soft body feathers a character likely to be desired in a fowl. The vastly increased size may have been a matter of selection, although, as the inhabitants of Shanghai feed their poultry but scantily, and, according to Mr. Fortune, mainly on paddy or unhusked rice, it is not easy to see how the size of the breed was obtained if, as is generally surmised, it arose from the little jungle fowl.
>
> Taking all these facts into consideration, I am induced to believe that the birds of the Cochin type did not descend from the same species as our game fowl. It may be asked what I would suggest as to the origin of these Eastern Asiatic breeds. In reply I would suggest the possibility, or even probability, of their being descended from some easily captured and really domesticated short-winged species that may have entirely passed into a state of domestication. . . .

These statements, though frequently quoted, have remained unchallenged and unsupported by any critical evidence for a considerable period of years. The senior author of the present report started several years ago to make a collection of skulls of different breeds and varieties of poultry with the object of checking upon the statements of Tegetmeier regarding the structural differences in the skulls. The focusing of attention in poultry breeding on relatively few breeds has resulted in some of the breeds in this collection becoming rare so that this series of skulls would be difficult to duplicate at the present time.

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The collection contains a total of 245 skulls, representing 27 breeds and varieties of chickens. All skulls are from birds near or beyond the age of sexual maturity. Measurements were made of the vertical and horizontal axes of the foramen magnum (occipital foramen), and observations were made of the nature of the suture between the two frontals.

STRUCTURE OF THE FRONTALS

From Tegetmeier's statements it would appear that he had observed that the Asiatic chickens had a much more prominent groove between

TABLE 1.—*Data on structure of frontal bones and foramen magnum in 27 varieties of chickens*

Variety	Sex	Condition of sulcus between frontals			Mean ratio of axes of the foramen magnum
		Grooved	Flat	Ridged	
American class:					
Jersey Black Giant	Male		2		1.15
	Female	1			1.11
New Hampshire	....do	2	3		1.15
Single-Comb Rhode Island Red	Male	10	4	1	1.15
	Female	26	14		1.15
Barred Plymouth Rock	Male	2	1		1.21
	Female	3	1		1.18
White Plymouth Rock	Male		1		1.15
	Female	2	2		1.16
White Wyandotte	Male	4			1.39
	Female	1	3	2	1.47
Silver Laced Wyandotte	....do	1			1.15
	Unknown	5	3		1.15
Asiatic class:					
Light Brahma	Male	1	2		1.31
	Female	4			1.12
	Unknown	1			1.15
Buff Cochin	Female	3			1.05
Black Cochin	....do	1			1.29
	Unknown	1			1.09
Partridge Cochin	Female	1			1.20
White Cochin	....do	4			1.10
English class:					
Australorp	Male	1			1.35
	Female	7			1.29
Dark Cornish	Male	1			1.38
	Female	3			1.17
	Unknown	2			1.25
Buff Orpington	Male	1	1		1.34
	Female	2			1.34
White Orpington	Male		1		1.11
	Female	1			.82
Mediterranean Class:					
Ancona	....do	2	1		1.17
Blue Andalusian	Male	2			1.30
	Female	2			1.12
Single-Comb Brown Leghorn	....do	1	9	1	1.18
Single-Comb Buff Leghorn	Male			2	1.15
	Female	6	4		1.19
Single-Comb White Leghorn	Male	1	1	3	1.22
	Female	6	11	3	1.13
Single-Comb Black Minorca	Male		1	3	1.22
	Female	2	6		1.16
Single-Comb Buff Minorca	Male		1	3	1.32
	Female			1	.96
Single-Comb White Minorca	Male			1	1.55
	Female	5			1.15
	Unknown	1			.88
Miscellaneous:					
Breda	Male		1		1.13
Round Head game	Female	1			1.32
Indian jungle fowl	....do		1		1.10
Barred Plymouth Rock-Rhode Island Red crossbred	....do	14	4		1.13
White Leghorn-Rhode Island Red crossbred	....do	5	8		1.19

the frontal bones of the skull than that found in other breeds of his day. It should be kept in mind, however, that several of the present-day breeds of the American and English classes were not known at the time he made his statements. Among the skulls examined in the present study were several of the Asiatic class, namely, the Light Brahma and the Buff, Black, Partridge, and White Cochins. Table 1 gives a list of the breeds and varieties studied, together with the quotient of the horizontal axis divided by the vertical axis of the foramen magnum. Mean ratios exceeding 1 indicate situations in which the horizontal axis is the longer and mean ratios of less than 1 indicate situations in which the vertical axis is the longer.

TABLE 2.—*Differences in classes of poultry in the structure of the frontal bones of the skull*

Breed group	Number of skulls	Percentage of frontals which are—				
		Extremely grooved	Moderately grooved	Slightly grooved	Flat	Ridged
American	94	3	17	40	36	3
Asiatic	18	28	39	22	11	
English	20	5	30	55	10	
Mediterranean	82	4	11	18	46	21

Table 2 shows the differences between four breed groups in the structure of the frontal bones of the skull. The data in table 2 show that the frontal bones may have grooves of varying depths, may be completely flat in that area, or may show a conspicuous ridge. (See fig. 1.) Since the numbers in each class differed widely, the distribu-
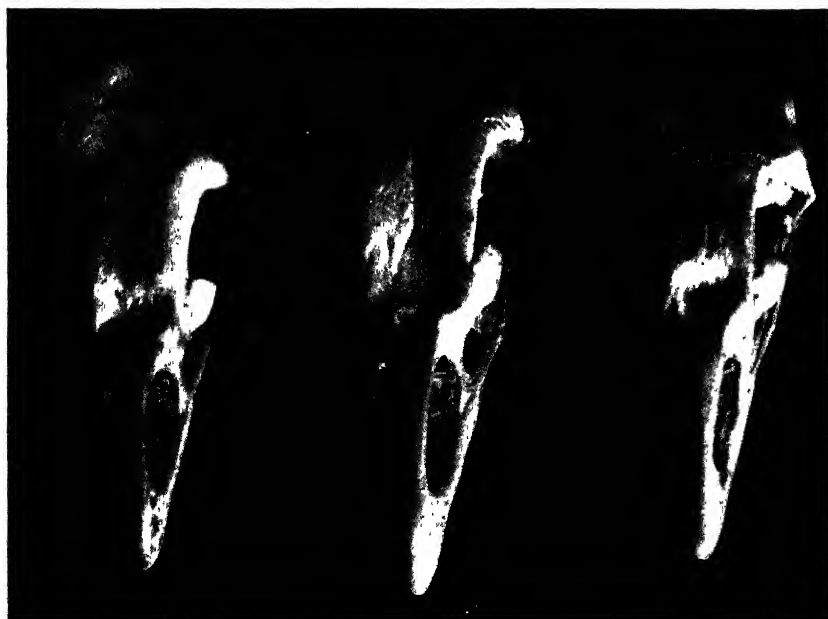
FIGURE 1.—Three skulls showing a flat, a deeply grooved, and a ridged suture between the paired frontal bones.

tion of the different types of frontals is expressed in percentage to facilitate comparisons, although it is recognized that some of the percentages are based on small numbers. The Asiatic groups not only showed a higher incidence of grooves between the frontals, but also possessed deeper grooves than the other groups.

The Mediterranean class showed less grooving between the frontals and a much higher incidence of ridges in the same area. Among the four breed and eight varietal representatives of the Mediterranean class, the Single-Comb White Leghorns and the Buff Minorcas showed the ridged frontals most conspicuously. A majority of the birds in the Mediterranean group failed to show any grooving of the frontals, which were either flat or ridged. The Asiatics had relatively few ungrooved frontals and were without ridges.

The representatives of the English class used in this study varied more in the direction of the Asiatics in lacking ridges and possessing grooved frontals. The grooving of the frontals of this group was less extreme than that of the Asiatics. The Dark Cornish had somewhat deeper grooves than the Australorps and Orpingtons.

The breeds and varieties of the American class were somewhat intermediate between the Asiatics and Mediterraneans with respect to the incidence of grooves and ridges. Of the seven varieties represented, the White Wyandottes varied most in the direction of the Asiatics.

In the miscellaneous group were females from the F_1 generation of crosses between the Barred Plymouth Rocks and Single-Comb Rhode Island Reds and also between Single-Comb White Leghorns and Single-Comb Rhode Island Reds. The pure Barred Plymouth Rocks and Single-Comb Rhode Island Reds did not differ much with regard to the frontal sulcus, and the crossbreds were similar to the parents. The Single-Comb Rhode Island Reds and the Single-Comb White Leghorns differed considerably in respect to the structure of the frontals, the Reds having only flat or grooved frontals with a majority grooved, while the Leghorns had more flat than grooved frontals and also some with ridges. The F_1 generation females from this cross showed only flat or grooved frontals wtih a majority of the latter type. No ridges appeared on the crossbreds, but the distribution of flats and grooves were more like that of the Leghorn, indicating dominance of the Leghorn type.

Table 3 presents an analysis of the relation of sex to the structure of the frontals. The representation of the two sexes was not equal, but the four classes of chickens had a fair number of each sex. In order to facilitate comparison, the distribution of the different types of frontals

TABLE 3.—*Relation of sex to skull structure*

Breed group	Number of skulls	Sex	Percentage of frontals which are—		
			Grooved	Flat	Ridged
American	25	Male	64	32	4
	61	Female	59	38	3
Asiatic	3	Male	33	67	
	13	Female	100		
English	5	Male	60	40	
	13	Female	100		
Mediterranean	19	Male	60	40	
	62	Female	100		

is expressed in terms of percentage. In all except the breeds in the American class, the females were exclusively in the group with grooved frontals. The males of all four classes had a considerable number with flat ungrooved frontals. This sexual dimorphism may have had some influence on the results shown in table 2, but in all classes of chickens represented, the number of female skulls greatly exceeded the males.

STRUCTURE OF THE FORAMEN MAGNUM

The vertical and horizontal axes of the foramen magnum were measured in all birds. This foramen, by which the spinal cord enters the skull, is somewhat variable in shape and dimensions. Its location and shape are shown in the posterior view of the skull given in figure 2. Owing to the sloping edges of the foramen, it is difficult to obtain exact measurements. In general, the practice was followed of taking the measurement at a point where it would give the maximum distance in the horizontal and vertical planes. The relative lengths of the axes were expressed by quotients of the horizontal axis divided by the vertical axis. Tegetmeier stated that the Asiatic chicken had its long axis in the vertical direction, and if expressed in the above-described manner, should have a value less than 1. Of the 245 individuals

FIGURE 2.—Posterior view of a skull showing the foramen magnum.

measured, only 9 had the longer axis of the foramen magnum in the vertical direction, and none of these was from the Asiatic class. A calculation of the mean ratios by classes shows the following results:

Class	Males	Females
American	1. 20	1. 19
Asiatic	1. 31	1. 17
English	1. 30	1. 27
Mediterranean	1. 28	1. 16

In the above calculations, individual ratios of less than 1 were eliminated, and these were usually found in females. The foregoing data, together with the observation that ratios of less than 1 were usually those of females, would indicate that the foramen magnum in females is proportionally narrower than in males. Though the differences were slight, the mean female ratio in each class was less than that of the males. It also cannot be said that the Asiatics differed markedly from the other classes. Thus the results found here would not support the statement of Tegetmeier that the Asiatics, as a group, differ significantly from other classes with regard to the relative dimensions of the foramen magnum (occipital foramen).

DISCUSSION

The observations of Tegetmeier, which he considered as evidence of polyphyletic origin of the breeds of fowl, though interesting, probably throw little light on the path of origin of the domesticated fowl. Evolutionists have long since abandoned the idea that similarities of structure between domesticated and wild species are critical evidence

as to the line of descent. The fact that the wild Indian jungle fowl has so many characteristics in common with some of our domesticated forms may indicate only that both have a relatively recent common ancestor.

The recorded history of poultry breeding procedures during the past century, in which most of our present-day breeds and varieties originated, indicates that there was little system or forethought involved. Any group of birds that appeared sufficiently different from existing breeds or varieties to permit identification was admitted to the official list, regardless of its qualities. It is interesting that despite this fact the Mediterranean class of chickens has such a distinctive set of traits, many of which were not set up as breed and varietal specifications. Small body size was a requirement, but other traits such as nervous temperament, large comb, white-shelled eggs, early feathering, and early sexual maturity were never prescribed characteristics of the breeds included in the Mediterranean class. It was earlier thought that there might be some physiological correlation of this group of traits found in the Mediterranean class; but more recent breeding accomplishments have demonstrated that at least early feathering, early maturity, and the white shell color may be combined with large body size. The observation here recorded that some of the varieties of the Mediterranean group have distinctive skull structures further supports the earlier evidence that this group of chickens has a genetic constitution which may have been drawn from an isolated pool of germ plasm.

It is not here contended that this evidence has any bearing on the early evolution of the domesticated chicken, but it may indicate something regarding the derivation of the more recent types of fowl. It is known that the earlier members of the Mediterranean class had their origin in Italy, while the foundation stock for our present day Asiatic breeds had its origin in importations from China and India. The early types were known as Cochin Chinas, Chittagons, Shanghais, and Brahma-pootras. The existing representatives of the American and English classes are known to have come largely from the early Asiatic importations, and these two classes have no special significance except to indicate source of origin. The lack of many of the traits of the Mediterranean group might be taken to indicate that there was not much infusion from this source into the constitution of the American and English classes. This view is further supported by the fact that the white varieties of the larger breeds are due to a recessive gene while the white of the Leghorn is due to a dominant gene.

The fact that the data in this paper do not support the statements of Tegetmeier does not necessarily mean that his observations were incorrect, since the stocks from the Asiatic group here examined were the results of approximately 60 years of selection beyond those available to Tegetmeier. It is of interest to note that the Mediterranean and Asiatic classes showed rather widely differing structure of the frontal bones of the skull when such differences were not observable in the living bird and could not have been intentionally modified by selection. Furthermore, the American and English groups were intermediate between the Asiatics and the Mediterraneans with regard to the structure of the frontal bones. The American and English classes are known to be of more recent origin than the Mediterranean and Asiatic classes and probably had their origin in stocks of these earlier established breeds.

SUMMARY

From these studies it is evident that there are rather wide differences in the structure of the skull at the point of union of the two frontal bones of breeds and varieties of poultry. The suture between the two frontals may be virtually smooth, sharply grooved, or ridged. There is also considerable variation in the depth of the groove. The representatives of the Asiatic and Mediterranean classes here studied showed considerable difference in the range of variation. Of the five classifications, extremely grooved, moderately grooved, slightly grooved, flat, and ridged, the Mediterraneans showed all types but relatively little grooving of any degree. The Mediterraneans had considerable ridging of the skull. The Asiatic group possessed no ridge but were distinguished by a high incidence of grooves, some of which was extremely deep. Although there was considerable overlapping of types of the two groups, they do show distinctive ranges of types. The Mediterraneans have relatively larger combs, but this would hardly seem to be responsible for the structural differences in the skull. No observed differences in skull structure lend support to the contention that the Mediterranean group is basically different in its origin from other groups. This study did not reveal any obvious differences in the structure of the foramen magnum as reported by Tegetmeier.

EFFECT OF INOCULATING A SOIL WITH AZOTOBACTER UPON PLANT GROWTH AND NITROGEN BALANCE [1]

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INTRODUCTION

In 1923 a 20-year experiment was begun in an effort to gain information on the following points: (1) The factors that influence the longevity of *Azotobacter* when introduced into a soil from which it is normally absent; (2) the effect of introducing and maintaining an *Azotobacter* flora in such a soil upon the crop-producing ability and the nitrogen balance of the soil.

The recent comprehensive survey of the literature on this subject by Allison [2] eliminates the necessity of a review of previous work; those interested in the problem are referred to this excellent summary.

METHODS

A nearly level and apparently uniform area of Geary silt loam, about 30 by 60 feet, lying at the summit of a gentle slope was selected for the experiment. The pH of the cultivated layer of this soil was in the vicinity of 5.8, and repeated tests for *Azotobacter* failed to reveal its presence.

Thirty-two 20-gauge sheet-metal cylinders, 3 feet long with a cross-sectional area of 5 square feet, were placed on the area, 6 feet apart from center to center in 4 rows east-west and 8 rows north-south. Prior to installation the cylinders were coated with asphalt paint and numbered from south to north, beginning at the southeastern corner. The soil was removed from an area slightly larger than the cylinder in four 8-inch layers and the cylinders were sunk into the soil 32 inches. Then each layer of soil, after being thoroughly stirred, was replaced in its original position.

Three months after installation the soil in the various cylinders received the following treatments, after which it was thoroughly spaded to a depth of 8 inches:

Series	Cylinders No.	Treatment
1	1 and 22	glucose.
2	2, 12, 18, and 28	inoculation with *Azotobacter*.
3	3, 9, 19, and 25	lime.
4	4, 10, 20, and 26	lime and inoculation with *Azotobacter*.
5	5, 15, 21, and 31	glucose, lime, and inoculation with *Azotobacter*.
6	6, 16, 17, and 27	no treatment (controls).
7	7, 13, 23, and 29	straw, lime, and inoculation with *Azotobacter*.
8	8, 14, 24, and 30	lime, inoculation with *Azotobacter*, and fallowed.
9	11 and 32	straw.

The straw (wheat), glucose (commercial), and lime (precipitated $CaCO_2$) were added at the rate of 2,000 pounds per acre. The inoculum consisted of 100 gm. of a mixture of several soils known to be abundantly supplied with *Azotobacter* naturally and, in addition, heavily inoculated with a mixture of crude laboratory cultures of *Azotobacter* obtained from a number of soils. The straw and glucose treatments were repeated annually following harvest, at which time all stubble and weeds were spaded into the soil.

Tests for available phosphorus, conducted on soil subsequent to treatment, indicated a possible deficiency. In 1925 and again in 1927 each cylinder received an application of superphosphate at the rate of 500 pounds per acre. In 1932 a second application of lime was given all soils receiving an initial lime treatment. At the same time all limed soils which received an initial inoculation with *Azotobacter* were reinoculated. The effect of this inoculation is evident in cylinder No. 6 (see table 1), which through error received the treatment intended for cylinder No. 5. Because of this error the data for cylinder 6 are not included in any calculations.

All cylinders and the surrounding area were seeded annually to a grain crop. The young plants in the cylinders of series 8 were pulled and spaded into the soil. Winter wheat was planted during the first few years, but it was found that a more uniform stand of oats could be obtained, and thereafter, with the exception of the final crop, oats were planted. Millet was planted the last season in the hope that any variations in the available nitrogen content resulting from treatment might be more strikingly reflected in the growth of a heavier feeding crop.

The crop was harvested by cutting just above ground and air-dried, and if the formation of grain seemed to justify its separation, the weight of seed as well as total weight was recorded. In some years the crop was so nearly a complete failure or suffered such serious damage from insects or rodents before harvest that yields were regarded as unsatisfactory for comparison. In such cases the crop was spaded into the soil. The total weight of 16 crops was regarded as satisfactory for comparative purposes; of these the grain weights of 12 were recorded separately.

On 15 occasions during the 20 years of the experiment the soil was sampled and tested for the presence of *Azotobacter*. Tests of numerous samples gave assurance that the pH value remained approximately 6.5 in the limed soils.

At the beginning and at the end of the experiment the soil of each cylinder was sampled in 8-inch sections for chemical analysis. The samples collected in 1923 were stored, and following completion of the experiment all samples were analyzed for total nitrogen by the Gunning method.

In cultivating the soil and in the collection of samples, those soils were handled first which, because of treatment or past observations, were regarded as being less likely to contain *Azotobacter*. This was the only precaution taken to prevent the transfer of *Azotobacter* from one cylinder to another.

During the first 10 years of the experiment, tests for *Azotobacter* were carried out by inoculating 50 ml. of a modified Ashby solution in a 300-ml. Erlenmeyer flask with the equivalent of 5 gm. of soil, incu-bating cultures at 28° C., and observing for the development of an *Azotobacter* film. This method was satisfactory for differentiating between the presence and absence of *Azotobacter* but gave little indi-cation as to the relative number present. Beginning with 1933, the same medium solidified with washed agar was poured into petri dishes and one-half gram of soil spread over the surface. The plates were incubated at 28° C. and observed for the development of *Azotobacter* colonies. The data recorded in table 2 for dates prior to 1933 are, therefore, not directly comparable with the data for later dates.

RESULTS

Data relative to the presence of *Azotobacter* in the variously treated soils throughout the experiment are recorded in table 1. Attention has been called to a difference in the method of testing for *Azotobacter* during the first and last halves of the experimental period. This differ-ence somewhat complicates a comparison of the data; nevertheless certain facts are clearly indicated:

(1) At no time did the uninoculated soil contain appreciable num-bers of *Azotobacter*.

(2) When *Azotobacter* alone was added to the soil it could be detected for a short period of time, after which it completely disappeared. (3) The application of phosphate and glucose or phosphate and wheat straw had no perceptible influence upon the longevity of introduced *Azotobacter*. (4) The application of lime alone so altered the soil as to make it capable of supporting *Azotobacter* when this organism was added (series 4) or accidentally gained entrance (compare cylin-ders 3 and 19 of series 3 with Nos. 9 and 25). Attention might be called to the relatively slow transfer of organisms from cylinder to cylinder; the soil in cylinders 3 and 19, for instance, had not become infected with *Azotobacter* after 20 years. (5) The addition of wheat straw or glucose, particularly the latter, along with phosphorus and lime made the soil a more favorable habitat for *Azotobacter* than did the lime alone. (6) The fallowed soil (series 8) served as a somewhat more favorable medium than a similarly treated cropped soil (series 4), possibly because of a more favorable moisture content.

Table 2 presents a summary of the data obtained relative to the influence of the various treatments upon the pH of the soil, the yields, and the nitrogen balance. It should be pointed out in connection with these data that in both yields and changes in the nitrogen content of the soil, differences between the cylinders in a given treatment were in many instances greater than the differences between the means of different treatments; hence no great significance can be attached to the recorded mean differences.

These data reveal that the pH of soils receiving no lime was be-tween 5.6 and 5.9, and that all limed soils were maintained at a pH of approximately 6.5 throughout the experiment. Treatments other than liming resulted in no significant alteration in pH value.

TABLE 1.—*Effect of various treatments of an Azotobacter—free soil upon the longevity of introduced Azotobacter*

Year and cylinder No.	Relative abundance of Azotobacter[1] in cylinder No. —																															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1924	—	4+	1+	4+	4+	—	4+	4+	—	4+	—	4+	4+	4+	4+	—	—	4+	—	4+	4+	—	4+	4+	—	4+	—	4+	4+	4+	4+	—
1925	—	4+	1+	4+	4+	—	4+	4+	?	4+	—	4+	4+	4+	4+	—	—	4+	—	4+	4+	—	4+	4+	1+	4+	—	4+	4+	4+	4+	—
1927	—	—	—	4+	4+	—	4+	4+	?	4+	—	?	4+	4+	4+	—	—	?	—	4+	4+	—	4+	4+	?	4+	—	—	4+	4+	4+	—
1929	—	—	—	4+	4+	—	4+	4+	—	4+	—	—	4+	4+	4+	—	—	—	—	3+	4+	—	4+	4+	—	2+	—	—	4+	4+	3+	—
1930	—	—	±	4+	4+	4+	2+	±	—	2+	—	—	2+	2+	4+	—	—	—	—	2+	4+	—	2+	2+	—	2+	±	—	2+	3+	4+	—
1933	—	—	±	1+	3+	—	3+	1+	1+	1+	—	—	±	2+	4+	—	—	—	—	1+	4+	—	2+	2+	—	1+	—	?	1+	2+	4+	—
1935	—	—	—	—	4+	—	2+	2+	±	1+	—	—	1+	3+	4+	—	—	—	—	2+	4+	—	2+	2+	2+	2+	—	—	—	2+	4+	—
1936	—	—	—	2+	4+	—	3+	2+	—	—	—	—	2+	2+	4+	—	—	—	—	1+	4+	—	1+	3+	2+	1+	—	—	1+	2+	4+	—
1937	—	1+	—	±	4+	—	4+	4+	—	1+	—	—	—	2+	4+	1+	—	1+	1+	2+	4+	—	2+	2+	2+	2+	—	—	2+	4+	4+	—
1938	—	—	—	—	2+	—	2+	2+	—	—	—	—	—	4+	4+	—	—	—	—	2+	4+	1+	1+	3+	2+	1+	—	—	1+	2+	4+	—
1939	—	—	—	2+	4+	—	2+	2+	1+	1+	—	—	2+	4+	4+	—	—	—	—	2+	4+	—	2+	2+	2+	2+	1+	—	1+	2+	4+	+
1940	—	—	—	2+	4+	—	4+	2+	2+	2+	—	—	2+	3+	4+	—	—	—	—	1+	4+	—	±	3+	2+	3+	—	—	2+	3+	4+	—
1941	—	1+	—	±	4+	—	4+	2+	2+	—	—	—	2+	2+	4+	—	—	—	—	—	4+	—	—	3+	2+	3+	—	—	2+	2+	4+	—
1943	—	—	—	—	—	—	4+	2+	2+	2+	—	—	—	2+	4+	—	—	—	—	—	4+	—	—	—	2+	3+	—	—	2+	—	—	—
Mean pH	5.79	5.72	6.50	6.66	6.79	—	6.62	6.48	6.52	6.58	5.94	5.75	6.60	6.57	6.72	5.77	5.78	5.77	6.57	6.58	6.60	5.90	6.01	6.49	6.39	6.63	5.87	5.80	6.62	6.54	6.66	5.83

[1] The relative number of Azotobacter is indicated as follows: — = None; ? = indefinite; 1+ = few; 2+ = some; 3+ = many; 4+ = abundant; ± = one of duplicate cultures negative.
[2] This cylinder of soil was inoculated through error just prior to this sampling.

TABLE 2.—*Effect of various treatments upon the pH, Azotobacter content, plant growth in and nitrogen balance of an Azotobacter-free Gary silt loam soil*

Series	Cylinders No.	Treatment	pH	Azotobacter content	Yield, dry weight		Change in nitrogen pounds
					Total	Grain	
					Grams	*Grams*	*Per acre*
6	16–17–27	None	5.8	None	99	35.9	−453
2	2–12–18–28	Inoculated	5.6	do	107	38.8	[1]−88(208)
3	3–9–19–25	Lime	6.5	Few toward end of experiment.	107	39.9	−176
4	4–10–20–26	Inoculated, lime	6.6	Few	105	38.3	[1]−557(403)
1	1–22	Glucose	5.8	None	101	34.6	−390
5	5–15–21–31	Inoculated, lime, glucose	6.7	Abundant	102	34.5	−208
9	11–32	Straw	5.9	None	116	40.2	+219
7	7–13–23–29	Inoculated, lime, straw	6.6	Some	117	42.4	+10
8	8–14–24–30	Inoculated, lime, fallow	6.5	Many			+29

[1] The values in parentheses are the means of the series when a single cylinder that deviated widely from the others is omitted.

The variability in plant growth from year to year and from cylinder to cylinder within the same treatment for any given year is apparent from the fact that the highest annual yield for any single cylinder occurred at least once and not more than four times for every treatment, including the control. Also, both the highest and lowest mean annual yields for any treatment occurred at least once in all but one treatment, and not more than four times for any single treatment. So variable were the yield data that when they were tested statistically the only treatment showing a significant increase over the control at the 5-percent level was the lime-straw-inoculated series. There is no evidence that this increase resulted from the inoculation, and probably should be attributed to nutrients, chiefly nitrogen, added in the straw during the 20-year period.

A somewhat comparable variability in the nitrogen balance for the 20-year period was also recorded. When these data were tested statistically the only treatment showing a significant loss at the 5-percent level was the control series. Despite the absence of statistically significant losses, the fact that the recorded data for the fallow and the two straw-treated series indicated no losses while the recorded data for all other treatments did indicate losses, seems highly suggestive. The fallow soil from which no crop was removed and on which erosion was prevented would not be expected to lose much nitrogen under the relatively low rainfall of this region, while the addition of the limited nitrogen in the straw would, in part, compensate for the nitrogen removed in the crop taken off such treated soils.

Another approach to the influence of the various treatments upon the nitrogen balance can be made by comparing the mean recorded gain or loss in nitrogen of one treatment with that of another to determine whether the difference between the two is significant. Since there were eight treatments exclusive of the fallowed series, which for obvious reasons is not comparable to the others, 28 such comparisons are possible.

The data shown in table 2 suggested that the differences between the mean change in the nitrogen content of treatments involving the application of straw (series 7 and 9) and treatments not including straw (series 1, 2, 3, 4, 5, and 6) would more likely be significant. When a statistical study was made of each of the 16 possible comparisons between 2 straw-treated series or between 2 treatments not including straw, in no instance was the difference between the mean recorded changes in the nitrogen content of the soils found to be significant at the 5-percent level. On the other hand, a similar comparison between straw-treated and non-straw-treated soils revealed that the differences were significant at the 5-percent level in 9 of the 12 possible comparisons, 3 of which were significant at the 1-percent level.

Two of the three comparisons between the straw vs. nonstraw treatments in which differences were not statistically significant involved the series (2) receiving inoculation alone. This might be interpreted as indicating an influence of the inoculum. However, the mere fact that the *Azotobacter* completely disappeared from the unlimed inoculated cylinders (series 2) in such a short period of time precludes the possibility of its exercising any influence upon the nitrogen balance of the soil over the 20-year period. It may be noted that the mean recorded loss of nitrogen from the inoculated series (2), was only 88 pounds, the low value being attributable to the change in one of the four cylinders deviating so markedly from that of the other three. If this cylinder were left out of consideration the mean loss would be 208 pounds. Similarly, the unusually high recorded loss of 557 pounds for the inoculated-limed series (4), was attributable to a single cylinder, which if left out of consideration, would reduce the mean loss in this instance to 403 pounds. No explanation is available as to why these two cylinders were so far out of line with others similarly treated. Their omission from consideration would bring these two treatments more nearly into line with other somewhat similar treatments.

In the preceding paragraph attention has been called to the virtual impossibility of the *Azotobacter* introduced into the soil of series 2 having any influence upon the nitrogen balance of the soil; thus this series might be regarded as a duplicate of the control series. This being true, the data recorded in table 2 relative to yields indicate that the application of straw was the only treatment giving even a suggestion of any influence of treatment upon yield. Actually the only instance in which any single treatment gave a significant increase at the 5 percent level over any other treatment was the inoculated-limed-straw series (7) over the control series (6). Again, this difference cannot be attributed to the presence of *Azotobacter*, since the application of straw alone (series 9) gave practically the same yield.

SUMMARY

The addition of superphosphate at the rate of 1,000 pounds per acre, or of the superphosphate and glucose or wheat straw at the rate of 2,000 pounds per acre annually, did not influence the longevity of *Azotobacter* introduced into a Geary silt loam, of pH value below

6.0, from which it was absent.   The addition of superphosphate and lime adequate to maintain a pH level of 6.5 enabled introduced *Azotobacter* to survive throughout the 20 years of the experiment. The suitability of this soil after liming as a habitat for *Azotobacter* was greatly enhanced by the annual application of a suitable carbohydrate food, i. e., glucose.   No evidence was obtained that the maintenance of *Azotobacter* in this soil for a period of 20 years influenced either the crop grown thereon or the nitrogen balance of the soil. The annual application of 2,000 pounds of wheat straw per acre did apparently influence favorably both plant growth and the nitrogen balance of the soil, but this cannot be attributed to the activity of *Azotobacter*.

THE EFFECT OF SUN, SHADE, AND OVEN-DRYING ON THE TOXICOLOGICAL AND CHEMICAL VALUES OF DERRIS ROOT [1]

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INTRODUCTION

Jones and Pagán (*3*) [2] recently demonstrated that there was no loss of rotenone when fresh derris roots (split or whole) were dried either in the sun, in shade, or in the oven. However, Jones et al. (*4*) and others (*6, 7*) have also shown that the toxicological value (rotenone equivalent) of derris roots is not perfectly correlated with the rotenone content. The data from these papers indicate that substances other than rotenone contributed to the toxicity of the roots. The fact that there was no loss of rotenone when derris roots were dried under the above condition does not indicate that there may be no loss in toxicity. A quick simple method for biologically determining the toxicity of derris roots has been developed by Pagán (*5*). Guppies (*Lebistes reticulatus* Peters) are used as test animals in this method and the toxicity assays are made in a relatively short time.

The guppy test has provided a convenient method for measuring the effect of sun, shade, and oven-drying on the toxicological value of derris roots. In this experiment various chemical measurements of toxicity including the red color value described by Jones (*2*), total chloroform extractives, and percent transmittance of acetone extracts suggested by Pagán and Loustalot (*6, 7*) were compared with the toxicological value (rotenone equivalent) of the roots dried in the sun, in the shade, and in the oven.

MATERIALS AND METHODS

The roots used in this experiment were from *Derris elliptica* (Wall.) Benth. variety Sarawak Creeping and were harvested from plants about 5 years old. Approximately 4,000 grams of washed fresh roots 4 to 10 millimeters in diameter were cut into 2- to 2.5-centimeter pieces. After cutting, the entire lot was thoroughly mixed and duplicate 300-gram samples weighed into tared containers and submitted to the following drying treatments until constant weight was obtained: (1) Whole roots were dried immediately in an air oven at 80° C. for 1 hour, then at 50° C. for 4 hours, after which the samples were placed in a desiccator for 4 hours and weighed, alternate drying in the oven

and the dessicator being repeated until constant weighing was obtained; (2) whole roots were dried in the sun; (3) root pieces were split longitudinally and dried in the sun; (4) whole roots were dried in the shade; and (5) split roots were dried in the shade. Samples of fresh root were taken at the outset and dried immediately at 105° to determine the initial moisture content.

When the roots in all treatments had reached constant weight subsamples were taken and dried at 105° C. to determine the final moisture content. They were then ground in a Wiley mill to pass an 80-mesh sieve and the toxicological and chemical values of the powdered roots determined. The rotenone content was determined by the method of the Association of Official Agricultural Chemists (1) and the chloroform extractives were determined from an aliquot of the chloroform extractives prepared for rotenone analysis. This aliquot was evaporated to dryness on a water bath and then in an oven at 105° C. for half-hour periods alternated with cooling in a desiccator over $CaCl_2$, until constant weight was attained. The red color (or rotenone plus rotenoids) value was determined by the modified red color test described by Jones (2). The transmittance value of the acetone extract of the roots was determined by the method described by Pagán and Loustalot (6), and the rotenone equivalent was determined with guppies as described by Pagán (5) Five replications of each treatment were used in the biological assays and the results were analyzed statistically.

RESULTS AND DISCUSSION

The roots dried in the oven reached constant weight within 1 day. Four days were required to dry whole roots in the sun in contrast to 6 days required for drying them in the shade. Sun-drying of split pieces was completed in 2 days, while 4 days were required to dry them in the shade. Weather conditions during the experimental period were more favorable for drying than in the previous experiment (3). The data presented in table 1 show that there was no loss of dry matter in whole roots dried in the oven or in whole or split roots dried in the sun. However, there was a small loss of dry matter in roots dried in the shade. This loss was somewhat greater in the whole roots which required 2 days longer to dry than the split roots. The losses in dry

TABLE 1.—*Effect of sun, shade, and oven-drying on the toxicological and chemical values of derris roots*

Drying treatment	Dry-matter losses	Rotenone	Toxicological value as rotenone equivalent [1]	Red color value	Total chloroform extract	Transmittance values of acetone extract at 300 mμ [2]
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Whole roots, dried in oven	None	6.4	11.3	14.6	18.9	34.5
Whole roots, dried in sun	---do---	6.3	13.5	14.7	18.5	34.2
Whole roots, dried in shade	2.1	6.3	16.9	14.9	18.8	33.6
Split roots, dried in sun	None	6.3	16.4	15.0	19.9	32.8
Split roots, dried in shade	.9	6.4	16.3	14.9	19.9	33.6

[1] The toxicological rotenone equivalent is the percentage of rotenone the root would need to contain in order to give the kill actually found.
[2] High values indicate low toxicity and vice versa.

matter are probably due to metabolic activity as suggested in the previous drying experiment (*3*).

The rotenone content of the roots was practically the same for all treatments. These results are in agreement with those reported by Jones and Pagán (*3*). The results of the biological assays also shown in table 1 revealed that the derris roots dried in the sun or shade, either split or whole, had a significantly higher toxicity expressed as rotenone equivalent than those oven-dried. Since the rotenone content of all samples was essentially the same, these data would indicate that some substance that adds to the toxicity of the root, other than rotenone, was lost or changed chemically during the period of oven-drying. Although the rotenone equivalent of whole roots dried in the sun was somewhat lower than that of split sun-dried roots or of whole and split roots dried in the shade, the difference was not statistically significant.

The red-color values were not markedly different in the roots of the various treatments, although there was some tendency for these values to be higher when the rotenone equivalent was high.

There was no statistically significant deviation in the total chloroform extractives of the various treatments. It is of interest to note that the percentage total chloroform extractives is greater in the roots that were split irrespective of sun or shade drying than in those of the other treatments. This may be due to the fact that the additional surface exposure of the fresh root produced by splitting afforded more opportunity for air oxidation of the root constituents. The percentage transmittance of acetone extracts of the roots correlate with the toxicity of the roots, much better than any of the other chemical assays.

SUMMARY

1. Fresh derris roots whole and split were dried in the sun, in shade, and in the oven until constant weight was attained. Whole roots in the oven were dried in less than 1 day. Whole roots dried in the sun required 4 days. Split roots were sun dried in 2 days while 4 days were required to dry them in the shade.

2. The rotenone content of roots of all treatments was practically the same, indicating that the drying treatments had no effect on this toxic constituent.

3. There was no statistically significant difference between the toxicological value of the roots dried in the sun or shade, whole or split. However, the rotenone equivalent of roots dried in the oven was significantly lower than those dried in the sun and shade. The fact that the rotenone content was the same in all roots indicates that some other constituents that contribute to the toxicity was destroyed or changed by the treatment.

4. The red color values were not appreciably different in any of the treatments, although there was a tendency for them to be somewhat higher when the rotenone equivalent was high. As found in the previous experiment, there was no statistically significant deviation in the total chloroform extractives of the roots from the various treatments. The percentage transmittance values of acetone extract of the roots gave the best correlation with the toxicological values.

5. These results indicate that derris roots may be dried either in sun or shade without appreciable loss in toxicity, but that drying

them in an air oven at 80° C.. then at 50° C. as in this experiment, results in a lower toxicological value even though there was no loss of rotenone.

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EFFECT OF ROOT DIAMETER ON CHEMICAL AND BIO-TOXIC CONSTITUENTS OF DERRIS [1]

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INTRODUCTION

The literature shows that there are different opinions as to the relationship between diameters and toxicological values of *Derris* roots. Moreau (*8*) [2] and Sievers (*12*) state that the large roots are generally inferior in quality as determined by their total extractives and rotenone content. In contrast, Worsley (*14*) reports an average rotenone content of 9.41 percent in "coarse" roots and 7.49 percent in fine roots. Koolhaas (*5*) in Java found that the extremely fine roots (1- to 2-mm. diameter) contained 9.0 percent rotenone while the large roots (10- to 17-mm. diameter) had but 4 percent. Moreau (*8*) states that in East Africa it has not been necessary to reject any of the roots from 3-year-old plants. This implies that even the large roots contained sufficient rotenone (5 percent) to be marketable.

Most workers in the field, however, believe that the medium roots, 4- to 10-mm. in diameter, are superior in rotenone content to the fine or large roots. The chemical data presented by different workers seem to support this point of view. The data reported by Maas (*6*) is typical. He found the following relationship between rotenone content and root diameter: Fine roots (below 2-mm. diameter) contained 5.3 percent rotenone, medium roots (2- to 6-mm.) 9.1 percent, and large roots (6- to 13-mm.) 7.9 percent. Moore (*7*) concluded that the medium root was superior in rotenone and total extractives. He found that in general the starch-bearing tissue predominated in the thick older roots and that the resin-cell tissue that contains the rotenoids was relatively more abundant in roots of small and medium diameters.

Since previous evaluations were based on the amount of rotenone or total extractives that the roots contained, it seemed desirable to assay the various root diameters biologically (*9*) and to compare these values with various chemical criteria.

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[1] Received for publication January 3, 1949.
[2] Italic numbers in parentheses refer to Literature Cited, p. 422.

METHODS AND PRESENTATION OF DATA

Two commercially important varieties of *Derris elliptica* (Wall.) Benth., Sarawak Creeping, and the MG clones (*4*) of the Changi variety, were studied in this experiment. Two replicates of 25 plants for each variety were harvested from border rows of a previous experiment. The Sarawak Creeping plants were 48 months old at time of harvest and the Changi-Mg clones 36 months. Because of this difference in age at harvest direct comparison between the two varieties cannot be made. The plants were excavated as completely as possible and the fresh roots were divided into the following diameter groups: 2 mm. or less, 2 to 4, 4 to 10, and over 10 mm. Many of the thick roots in the Sarawak Creeping variety were over 25 mm., which is much larger than ordinarily found in roots harvested at 26 to 32 months. In the MG clones the maximum root diameter was around 15 mm., which is normal. All the roots were dried in the sun; the thicker roots were split to hasten drying. The air-dry weights of the different sizes in each variety are given in table 1.

TABLE 1.—*The yield of roots and their toxic constituents obtained by a diameter classification of 2 varieties of Derris elliptica, MG and Sarawak Creeping (50 plants each)*

	Yield of roots		Yield of rotenone [1]		Yield of rotenone equivalent [2]	
	Grams	*Percent*	*Grams*	*Percent*	*Grams*	*Percent of total*
MG clones·						
Less than 2 mm	165. 0	4. 4	13. 5	4. 5	14 2	4. 1
2-4 mm	447 0	12 0	49. 2	16. 6	54. 9	15. 6
4-10 mm	2, 299. 0	61. 9	197.7	66. 5	241 0	69 1
10+ mm	802. 0	21. 7	36 9	12. 4	39 3	11. 2
Total	3, 713. 0	_____	297.3	[3] 8 0	349 4	[4] 9. 4
Sarawak Creeping						
Less than 2 mm	408. 5	6. 4	15. 1	6. 0	22. 4	6. 7
2-4 mm	1, 423. 5	17. 6	65. 2	25. 9	90. 5	27. 0
4-10 mm	2, 472. 0	38. 7	133. 5	53 0	222 6	66. 3
10+ mm	2, 385. 0	37. 3	38. 2	15. 1	0	0
Total	6, 389. 0	_____	252. 0	[3] 3. 9	335. 5	[4] 5. 3

[1] Rotenone yield obtained by multiplying the percentage of rotenone determined chemically by the yield of root (gram) of each of the 4 diameter sizes.
[2] Rotenone equivalent yield determined by multiplying the percentage rotenone equivalent obtained by the guppy test by the yield of root (gram) of each of the 4 diameter sizes.
[3] Obtained by dividing total rotenone in grams by total yield of roots (gram).
[4] Obtained by dividing total rotenone equivalent in grams by total yield of roots (gram).

In the MG clones the bulk of the root, about 62 percent, consisted of medium roots of 4 to 10 mm. in diameter. The rest of the yield was distributed as follows: 4 percent in the 0- to 2-mm. group, 12 percent were 2 to 4 mm., and 22 percent were over 10 mm. in diameter. The air-dry yield per plant was 74.2 gm. and the total yield for the 50 plants 3,713 gm.

The distribution by diameter groups of the Sarawak Creeping roots followed an entirely different pattern. Roots of the 0- to 2-mm. size yielded 6 percent of the total, while 17 percent were found to be in the 2- to 4-mm. class. The two largest diameter groups, 4 to 10 and 10+ mm. yielded 38 and 37 percent, respectively. These data agree fairly well with the results obtained by White, Pagan, and Jones (*13*). The yield per plant in the Sarawak Creeping variety was 127.7 gm. and the total yield for the 50 plants 6,389 gm.

The roots were all ground to pass a 0.5-mm. sieve and analyses were made for rotenone (*1*), red-color value (*2*), total chloroform extractives, and transmittance (*10*). The results from these determinations are presented in table 2. The biological assay was made by the "guppy" method (*9*). In order to facilitate statistical analysis between the different root sizes, the same amount of root powder (0.250 mg.) was weighed in all cases. A composite sample of all roots of each of the varieties was included in the tests. These composites were made by mixing the different root diameters on the basis of weight percentages of their respective yield of air-dry roots. The toxicological data is presented in table 3.

TABLE 2.—*Chemical analyses by root diameters of 2 varieties of Derris*

Derris elliptica	Root diameter	Rotenone [1]	Total CHCl₃ extractives [2]	Red color value [3]	Transmittance
	Millimeters	*Percent*	*Percent*	*Percent*	*Percent*
MG clone	0-2	8 2	19. 5	17. 5	52 5
	2-4	11.0	26. 5	23. 5	48. 5
	4-10	8.6	20. 3	18. 1	56. 0
	10+	4.6	10. 2	9.0	70. 5
	Composite	7.0	17.3	15. 0	53. 0
Sarawak Creeping	0-2	3.7	10. 6	9. 5	63. 0
	2-4	5 8	15.3	14. 0	57. 5
	4-10	5. 4	15.7	12. 5	58. 0
	10+	1.6	5. 2	4. 6	79. 0
	Composite	4. 1	10. 9	10. 0	61. 0

[1] Differences between rotenone percentages greater than 0.89 percent are significant at odds of 99:1.
[2] Differences between total chloroform extractives percentages greater than 2.6 percent are significant at odds of 99:1.
[3] Differences between red-color value percentages greater than 2.2 percent are significant at odds of 99:1

TABLE 3.—*Results of toxicological assay by root diameters of 2 varieties of Derris*

Variety of *Derris elliptica*	Root diameter	Test solution [1] rotenone	Mean [3] mortality	Standard error	Rotenone equivalent [2] Test solution	Rotenone equivalent [2] Root	Increase in toxicity due to rotenoids
		Milli- grams per milliliter	*Percent*	*Percent*	*Milli- grams per milliliter*	*Percent*	*Percent*
MG clone	0-2	0.082	50. 0	4. 1	0.086	8. 6	4.8
	2-4	.110	72. 5	2. 5	.122	12. 2	10. 9
	4-10	.086	62. 5	6 3	.105	10. 5	22. 0
	10+	.046	20. 0	7. 1	.049	4. 9	6. 5
	Composite [4]	.070	67. 5	11. 1	.112	11. 2	59. 9
Sarawak Creeping	0-2	.037	22. 5	8 5	.055	5. 5	48. 6
	2-4	.058	47. 5	7. 5	.081	8. 1	39. 6
	4-10	.054	55. 0	5. 0	.090	9. 0	66. 5
	10+	.016	0	0	0	0	0
	Composite [4]	.041	25. 0	6. 5	.054	5. 4	31. 7
Rotenone:							
Standard 1		.050	22. 5	7. 5			
Standard 2		.100	62. 5	9. 5			
Standard 3		.200	90. 0	4. 1			

[1] Concentration of root powder in test solution was in all cases 1.0 mg. per ml. of acetone.
[3] Differences between mean mortalities of 9.7 per cent were significant at odds of 19:1.
[2] Rotenone equivalent is the amount of rotenone the root must contain in order to give the kill actually found.
[4] Compounded on a percentage weight basis to represent the entire root and analyzed separately.

Based on the results of the biological assays, the diameter groups 2 to 4, and 4 to 10 mm. in Sarawak Creeping were the most toxic for this variety and both were superior by high significance to roots less than 2 mm. and those over 10 mm. in diameter. Roots less than 2 mm. in diameter ranked third in toxicity while the largest roots were the least toxic. The thick roots (over 10 mm. in diameter) produced no mortality in the guppy test. The amount of rotenone plus the unknown amount of rotenoids present in the 250-mg. sample used in the biological tests did not give an extract of sufficient concentration to reach the toxicity threshold. A toxicity value could have been established for the large diameter roots by increasing the amount of powder used for the test. This was not done because the statistical treatment of the data required a constant weight of sample for each of the diameter groups. It is interesting to note that nearly 35 percent of the total yield of dry Sarawak Creeping roots belonged to this group of large diameter roots.

The bio-assay of the MG clones gave results entirely different from those obtained with Sarawak Creeping. Roots with diameters from 2 to 4 mm. were significantly better than the other three groups. Roots in the 4- to 10-mm. class came next, followed closely by 0- to 2-mm. group. These two groups were significantly better than the roots 10 mm. in diameter and larger.

The rotenone equivalent for the composite sample of Sarawak Creeping was found to be 5.4 percent; as shown in table 1 the value calculated from the individual percentage rotenone equivalents of the diameter groups was 5.3 percent. This good agreement between the calculated and the observed toxicity of Sarawak Creeping roots is in sharp contrast with the values found in the MG clones. In this variety the calculated rotenone equivalent was 9.4 percent and the actual value as determined on guppies was 11.2 percent. The reason for this disagreement is not known.

Table 1 also presents a comparison of the yield of rotenone equivalent by diameter groups. The yield was obtained by multiplying the mean yield of air-dry root for each diameter by the percentage of rotenone equivalent obtained with the guppies. These results show that even though the best quality roots of the MG clones were in the 2- to 4-mm. diameter group and the best quality Sarawak Creeping roots were in the 4- to 10-mm. group, the highest yield in both cases fell in the 4- to 10-mm. group.

The relation between chemical and toxicological constituents is shown graphically in figure 1. In this figure the different chemical constituents and the rotenone equivalent were plotted against the root diameter. The similarity of the shape of the lines show that in both Sarawak Creeping and MG clones there is a direct relationship between the chemical values and the actual toxicity. The total chloroform extractives in both varieties correlated more closely with rotenone equivalent than did any of the other chemical constituents. This can be seen also from the ratios presented in table 4 and substantiates experiments previously reported (*11*). The transmittance values obtained from the Sarawak Creeping plants correlate quite well with the bio-assay, while in the MG clones this value does not agree with the rotenone equivalent as well as the other chemical constituents.
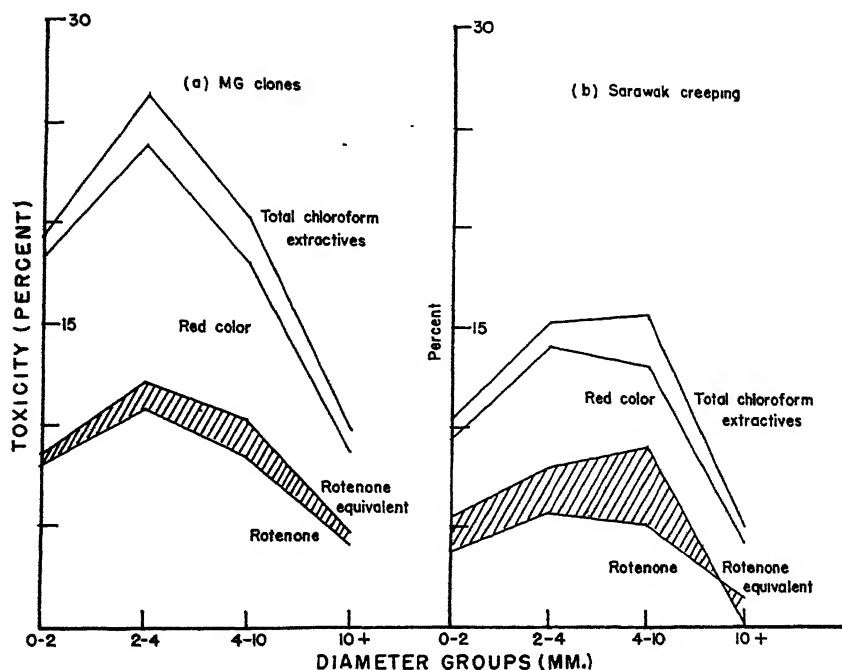
FIGURE 1.—Chemical and toxicological values of some derris roots plotted against root diameters.

TABLE 4.—*Comparison of chemical and toxicological values by diameter groups of 2 varieties of derris root*

Sample	Ratio of rotenone equivalent to—		
	Total CHCl₃ extractives	Red color value	Rotenone
MG clones:			
Less than 2 mm	0. 440	0. 491	1. 05
2–4 mm	. 460	. 519	1. 11
4–10 mm	. 517	. 580	1. 22
10+ mm	. 480	. 544	1. 06
Sarawak Creeping:			
Less than 2 mm	. 519	. 578	1. 48
2–4 mm	. 529	. 578	1. 40
4–10 mm	. 573	. 720	2. 00
10+ mm			

The increase in toxicity due to rotenoids is very noticeable in Sarawak Creeping and less so in MG clones, as shown by the shaded area in figure 1. This is in agreement with the work of Jones et al (*3*). In both varieties the rotenoids in 4- to 10-mm. roots were most toxic, causing an increase in toxicity of 66.5 percent in Sarawak Creeping and of 22.0 percent in MG clones.

DISCUSSION

The rotenone content in the roots of the composite sample of Sarawak Creeping was 4.1 percent. This is below the minimum marketable standard of 5 percent rotenone. However, if the thick roots (10-mm. and over), which contain only 1.6 percent rotenone, are not included in the composite the average rotenone content of the root system increases from 4.1 to 5.3 percent and the average rotenone equivalent increases from 5.4 to 8.4 percent. Since the thick roots represent 33.0 percent of the total root yield and contain 15.0 percent of the total rotenone content, a considerable loss of toxic ingredients would result by discarding this material. Since a proportionate increase of the large roots lowers the quantity of rotenone, it would appear that harvesting of this variety would be most profitable if done before the large roots develop. This contention is supported by the work of White et al (*13*) which indicates that composite root samples of Sarawak Creeping contain 6.6 percent rotenone at 26 months and 5.6 percent at 32 months.

SUMMARY

The data presented show that in the MG clones roots 2 to 4 mm. in diameter were the best in quality, while roots 4 to 10 mm. in diameter gave the best yield and contained the greatest amount of toxic constituents. Furthermore, considering the age of the plant and local growth conditions, all of the root produced by this variety was of marketable quality. In the Sarawak Creeping variety the group 4 to 10 mm. in diameter was the best in quality and contained the greatest quantity of toxic constituents. Roots less than 2 mm. or above 10 mm. in diameter do not have the minimum rotenone content for marketable roots; rejecting the thick roots (10 mm. and over) would bring the remaining roots to marketable standard.

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CRITERIA FOR TESTING VANILLA IN RELATION TO KILLING AND CURING METHODS [1]

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INTRODUCTION

The widely known flavor, vanilla, is prepared from the vanilla bean which is the fruit of a tropical orchid, *Vanilla fragrans* (Salisb.) Ames. Actually, the fruit is not a bean but a pod, 5 to 10 inches long, one-quarter to three-quarters of an inch in diameter, and somewhat triangular in cross section. Inside the thick fleshy outer wall are numerous small black seeds. The pods are harvested from the plant when a yellow color develops on the blossom end of the pod. They are then cured in four steps as follows:

(1) Killing—by wilting, or killing by immersion in hot water or by sunning;

(2) Sweating—by exposure to the sun and wrapping in blankets, or warming in an oven;

(3) Drying (the beans now dark brown and flexible) to 15 to 30 percent;

(4) Conditioning—storing the product for several months in closed boxes at room temperature, during which time the flavor develops.

The cured material has the characteristic vanilla aroma and is ready for market.

For use as a flavor, the beans are ground with sugar and other ingredients or extracted with alcohol or some other nontoxic solvent to obtain the well-known vanilla extract. To enhance its flavoring value, the final product is often fortified with vanillin prepared from lignin or from clove oil.

CRITERIA FOR TESTING VANILLA

It is generally agreed that the criteria usually applied to vanilla do not constitute a true measure of the quality of the product. An analysis of vanillin and determination of phenol value, lead number, and resin content may be useful in identifying vanilla and detecting adulteration, but they are of little value in measuring quality. Even the usual grader's examination for texture, color, aroma, and appearance, which is valuable in obtaining rapid estimation of quality, is not entirely satisfactory. Each of these properties is only symptomatic of quality; that is, the product may appear to be excellent according to several of these criteria and yet may be quite inferior when used as a flavor. Or, two samples may appear fairly equal according to these tests and still differ markedly in flavoring strength and other characteristics.

Vanilla, as well as other similar materials, can be tested organoleptically in ice cream by several testers and the results analyzed statistically to evaluate the degree of difference among the samples. This method was tried on a set of extracts, to evaluate both the products and the techniques of testing.[3]

The same extracts were used numerous times at various strengths and in different recipes. The most satisfactory ice cream recipe to date calls for 1 pint of cream (40-percent butterfat), 4 pints of milk (4-percent butterfat), 15 percent sugar, and 0.3 percent gum tragacanth. The mix was divided into separate portions, one for each sample and the extract added at the rate of 2 cc. per 100 cc. of mix. To one sample in each series, the blank, no extract was added. After cooling to 10° C. the samples were churned in a hand freezer to a hard consistency and stored at −10° until tested.

The vanilla extracts used throughout these curing experiments were prepared as follows: The moisture content of the cured beans was determined so that the extracts could be made with a known proportion of alcohol to dry vanilla tissue. The beans were cut to 1-cm. slices and soaked in 10 cc. of 50-percent alcohol per gram of dry matter. The beans and alcohol were kept in a flask fitted with an air condenser. Over a period of 2 weeks the flasks were warmed twice daily in a water bath at 60° C. At the end of this time, the extracts were filtered through cotton to remove the solids.

The ice cream samples were tasted from 3 to 22 hours after freezing in two series with coded designations. The ranking of each tester was checked for reliability by calculating the correlation coefficient between the two separate rankings made by the same tester. The data were accepted as reliable if the tester obtained the same ranking both times, or if in the second run he interchanged two samples other than the blank that were consecutive in the first run. However, if the tester interchanged first and third rank, the correlation coefficient, then below 0.77, was considered too low and the tester's data were rejected. Using the ranks obtained by reliable testers, the

[3] BLISS, C. I., ANDERSON, E. O., and MARLAND, R. E. A TECHNIQUE FOR TESTING CONSUMER PREFERENCES, WITH SPECIAL REFERENCE TO THE CONSTITUENTS OF ICE CREAM. Connecticut [Storrs] Agr. Expt. Sta. Bul. 251, 20 pp. 1943.

data were then treated statistically as though each ranking were made by a different tester. For example, if three reliable testers each made two acceptable determinations, the final statistical analysis was made as though there were six testers.

The same set of extracts was used on several different dates and scores for the samples calculated each time. If, on any given date, the differences between scores were not considerably greater than that necessary for high significance, the data for that date were discarded on the assumption that the recipe used was such that the differences between samples were not brought out sharply. Of course, if this occurred consistently, the data were accepted and the conclusion drawn that the differences among the samples were not great. However, where appreciable differences were obtained on some dates, the data from runs in which differences were small were discarded and the weakness of the data attributed to the receipe.

COMPARISON OF KILLING METHODS

In 1943 an experiment in which different curing methods were compared showed that scratching the beans was a good way to kill them.[1] Recently, further trials were made with combinations of the following killing methods singly and in combination: (1) Dipping in water at 80° C. three times for 10 seconds at 30-second intervals; (2) scratching each face 1 mm. deep from end to end (some beans were scratched only 2 cm. from the stem end to decrease the brittle nature of the stem end in the cured product); and (3) frozen at −10° for 24 hours. After killing the samples were oven-sweated at 45° and dried at room temperature to 28.6 percent of the fresh weight, after which they were conditioned in closed boxes at room temperature for 6 months. Duplicate 260-gm. samples of 6- to 8-inch blossom-end-yellow beans were used. No mold developed in any of the samples and splitting was negligible.

Those killed with hot water were smooth, oily, and dark brown during sweating. During drying the oiliness changed to stickiness and a very slight vanilla aroma became noticeable. The conditioned product was very dark brown, somewhat rough, and not flaccid. The beans were shiny and had no vanillin crystals on the outside. The aroma was prunelike with strong vanilla character.

Beans killed by scratching required about 1 day to become brown, which was somewhat longer than in the other treatments. The beans were oily during sweating, but somewhat less after drying. Some flowery aroma developed during drying. Those scratched to 2 cm. from the stem end had a firm lump in this position which later disappeared and left the stem end flexible. Those scratched along the entire length became woody at the stem end. The texture was more flaccid than those killed with hot water but was nevertheless firm. After conditioning, the color was dark brown with some reddish cast and the oiliness had subsided. The aroma was strong, flowery, very agreeable, and fully developed. Of all the treatments, only this one resulted in vanillin crystals being formed on the surface of the beans. It was also noted that the samples were completely cured in about

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[1] ARANA F. E. VANILLA CURING AND ITS CHEMISTRY. Puerto Rico (Mayaguez) Agr. Expt. Sta. Bul. 42, 17 pp., illus. 1944.

2 months of conditioning. Those scratched the entire length of the bean had woody stem ends, but those scratched to 2 cm. from the stem end did not.

The combination treatments consisting of a hot-water kill followed by scratching, as well as that consisting of scratching followed by a hot-water kill, resulted in a product similar to that of hot-water kill alone. However, the vanilla aroma was not so strong in the scratched beans, and plugs of resin formed in the scratches which lent a fermented aroma to the beans.

The samples killed by freezing were characterized by a reddish-brown color and a very flaccid texture that persisted throughout the curing process. During sweating the beans were slightly oily, but this had subsided by the time conditioning was over. The product was smooth and of sweet, suave aroma with less vanilla character than those killed by the hot-water or scratching process.

The combination treatments, hot water-freeze, freeze-hot water, scratch-freeze, and freeze-scratch, resulted in a product like that of frozen beans except that the aroma was a little stronger in the double-treated samples. The color was reddish brown and the aroma sweet, similar to beans killed by freezing. Of the combinations the scratch-freeze process resulted in the strongest vanilla aroma.

According to the foregoing observations on aroma the three single-killing processes ranked as follows: First, scratching; second, hot water; third, freezing. The scratching method was best when the scratch was not carried all the way to the stem end; woody stem ends were thus avoided. None of the combination procedures were out-standing improvements over the single methods. However, the freezing method was improved to some extent when it was preceded by a scratching or a hot-water kill.

Extracts were prepared from vanilla beans killed by different methods, and the vanillin analyses on a dry-weight basis gave the following results: Hot-water killing method, 2.80 percent; scratched, 3.30; frozen, 2.86; hot water, scratched, 2.85; scratched, hot water, 2.86; hot water, frozen, 3.00; frozen, hot water, 2.66; scratched, frozen, 2.86; and frozen scratched, 1.93 percent.

Scratching the beans resulted in the highest vanillin content and it was previously noted that the product appeared to be superior. However, organoleptic tests made with ice cream prepared from the extracts indicated that the extract made from beans killed by hot water scored best and those killed by freezing scored second. The difference between the two was not significant. Both were superior by high significance to the extract of beans killed by scratching. The latter was significantly superior to the blank. In appearance, the scratched beans seemed to be superior because of the rapid rate of curing, vanil-lin crystallization, high vanillin content, and good aroma. However, according to the organoleptic ice cream test, which is a more objective criterion, the beans killed by hot water and freezing were superior. This indicates again that the appearance criteria are not necessarily related to final quality.

EXPERIMENTAL CURING METHODS

WHOLE, CUT, AND GROUND BEANS

In the following work the above criterion of vanilla quality was used in some cases in conjunction with the ordinary tests. The curing of vanilla using whole, cut,. and ground beans has been the subject of extensive investigation at this station. A previous report [4] presented the results obtained in experimental curing procedures as well as in biochemical studies of the enzymatic processes involved in curing.[5] These experiments emphasized the importance of oxidative changes that occur during curing. It was found that the principal oxidizing reactions of the vanilla enzyme system were brought about by an oxidase. That this oxidase could also oxidize vanillin and other phenols has been determined. Thus it becomes of interest to study the role of oxygen in the process. The present experiment was designed primarily to show the effects of aeration on the bean tissue during curing. A second objective was to determine whether such a method could be used to simplify the curing methods commonly used.

Duplicate 200-gm. samples of fresh beans, harvested when the blossom end became yellow, were used in four different degrees of fineness: (1) Whole, (2) 1-cm. slices, (3) ground through a food chopper, and (4) ground with a pestle with sand in a mortar. The main difference between treatments (3) and (4) was that, in the former, few cells would be ruptured while in the latter most cells would be broken. The samples were then oven-killed at 60° C. for 24 hours, sweated until the whole beans were flexible, dried to 28.6 percent of the fresh weight, and conditioned in closed jars at room temperature.   •

The whole beans became dark brown, oily, and developed a suave, flowery vanilla aroma. The beans cut to 1-cm. slices became dark brown and shiny, but showed a green undeveloped character which, during conditioning, turned to a prunelike aroma; vanilla aroma was weak. Samples ground in the food chopper turned brown on top where exposed to air, but became dark brown throughout only after mixing. The aroma was like that of the cut beans except that the prunelike note was even stronger; in fact, it was so strong that no vanilla aroma could be detected. Beans crushed in the mortar also turned brown on top where exposed to air and had to be mixed to obtain a uniformly dark brown mass. While drying, a slight vanilla aroma developed, somewhat like that of the controls but not so strong.

Extracts were prepared from these samples for vanillin analysis and organoleptic tests. It was found that beans cured whole contained 3.45 percent of vanillin, those cut to 1-cm. slices 3.21 percent, those ground in the food chopper 3.65 percent, and those ground with sand 3.79 percent. Apparently the sliced beans lost some vanillin, probably by sublimation, while the ground beans had even more vanillin than the controls because of the intimate contact brought about between the glucovanillin and the hydrolyzing enzyme and because

[4] ARANA, F. E.   VANILLA CURING AND ITS CHEMISTRY.   Puerto Rico (Mayaguez) Agr. Expt. Sta. Bul. 42, 17 pp., illus. 1944.
[5] See second paper of this series in the Journal of Agricultural Research, vol. 78, No. 11, for further results in enzyme studies.

of the decreased exposure during which vanillin could sublime. Organoleptic tests made with ice cream showed no differences among the products of these four treatments.

After organoleptic examination of these extracts, Mr. Rosenbaum made the following comments: The extract of the beans cured whole, No. 5, had "a full bodied aroma, a rich Mexican type characteristic well rounded. The best of No. 5 to 10." The extract of the beans cured in slices, No. 6, was "clean and fairly well rounded; more flowery than No. 5, and somewhat lighter in depth and character." The extract of the beans passed through a food chopper, No. 7, was "well rounded with a full-bodied aroma that was close to No. 5 in all characteristics, but seemed to be slightly less full than No. 5." The extract of the beans crushed with sand, No. 8, was "more insipid than Nos. 5, 6, and 7; slightly flowery in character. Lacks well rounded body. Similar to No. 6 generally, but apparently not as well developed." He regarded the products from whole beans and from beans ground in a food chopper as the best.

It is concluded from this experiment that excessive aeration such as that obtained with cut or coarsely ground beans resulted in excess oxidation and a consequent development of a prunelike note in the aroma. Because of the simplicity of curing chopped vanilla these processes appear promising. In spite of the differences in vanillin content, aroma, and color, it is interesting to note that little difference in the flavor of the extracts could be demonstrated.

CURING THE SEPARATE PARTS OF THE FRUIT

Two-hundred-gram samples of beans were sectioned longitudinally to give one sample of the pod wall, or fleshy part of the bean, and another sample consisting of the central seed portion with placental tissue. On curing, the seed and placental tissue turned brown but developed no aromatic character. The pod wall without central seed portion and placental tissue turned brown and developed a strong sweet aroma with some vanilla character during the sweating and drying period. These samples were especially oily inside where the seeds had been removed. The oil became sticky and finally hardened to a resinous film. During conditioning a fermented acid note became noticeable, but vanilla aroma was still present. After conditioning, the vanilla character predominated; however, this character was incomplete as compared to whole cured beans.

Extracts were prepared from these samples for vanillin analysis and organoleptic tests. The seed and placental tissue contained 2 percent vanillin, dry basis, but when the tissue was incubated with emulsin the percentage increased to 2.74, indicating that unhydrolyzed glucovanillin was present. The cured outer wall contained 4.03 percent of vanillin. From the fresh and final weights and the vanillin and moisture contents it was calculated that the outer portion constituted 64.7 percent of the dry matter of the beans and contained 72.8 percent of the vanillin. The remainder in the seed and placental tissue was 35.3 percent of the dry matter and 27.2 percent of vanillin.

Concerning these extracts Mr. Rosenbaum said: The extract of the seed and placental portion has "a very heavy character with a strong

flowery note and a distinct Mexican end aroma. A well rounded extract." The extract of the outer portion has "a fatty odor seemingly somewhat scorched in character. Almost lacking in sweet note but has a heavy Mexican type end product. Not well rounded at all." The extract of whole beans from this same lot was considered to have a "full bodied aroma with a rich Mexican type characteristic; well rounded; best" of samples submitted.

CONDITIONING TEMPERATURES

Previous experimental work in vanilla curing has involved different methods of killing, sweating, and drying but no attention has been given to the final stage, conditioning. It is recognized that considerable change takes place during the initial stages of curing but the principal development of aroma occurs during conditioning. It is therefore of interest to condition the product in different ways to determine whether the final product is affected. One variable that is readily changed is the temperature at which conditioning is carried out. In commercial curing, if warm temperature were desired, conditioning could be done in a loft or a sun-heated shed. If cool temperature were desired, conditioning could be carried out in a basement or a similar cool place.

To determine the effect of temperature during the conditioning stage, two trials were made. In the first trial three lots of 250 gm. each of blossom-end-yellow beans were cured as follows: The beans were frozen solid overnight at $-10°$ C., thawed and dipped in hot water (80°) for three 10-second periods at 30-second intervals. After sweating and drying to 30 percent of the fresh weight, conditioning was carried out in closed cans at three temperatures, 13°, 27°, and 35°. Weekly examinations were made to observe weights, aroma, color, vanillin crystallization, and mold development.

During the curing process no mold developed on any of the samples and no differences in color appeared among the treatments. Within 1 month after the beginning of the conditioning period, the beans at 35° C. had developed a slight aroma. Subsequently the aroma of these warm-conditioned beans developed more fully and had a pungent note characteristic of Mexican vanilla. Those kept at room temperature were characterized by a more flowery aroma typical of Puerto Rican vanilla and eventually the aroma was more developed than was the case with those conditioned at 13°. However, the cold-conditioned beans had a sweet odor early in the experiment before either of the other groups developed a definite aroma.

Vanillin crystals appeared after 6 months' conditioning on the beans kept at low temperature. Those at room temperature had crystals within 10 days, but those conditioned in the oven produced no crystals.

Samples for tasting were taken 6 months after conditioning was started. In all samples the seeds were tasteless and sandy. Those kept at the lowest temperature had the most pleasant aroma but, as the conditioning temperature increased, the aroma was stronger. The outer wall of those kept at 35° C. was bitter and aromatic and had a taste similar to that of chewing tobacco.

After 7½ months of conditioning, moisture determination showed that beans kept at low temperature contained 27.9 percent of moisture, those kept at room temperature 21.9, and those in the oven 15.8 percent. Vanillin analysis by the method used by the Association of Official Agricultural Chemists showed that on the dry basis all the samples contained about 3.5 percent vanillin.

Ice cream samples were made with the extracts from the lots of vanilla beans that had been conditioned at the three different temperatures. The samples from the beans conditioned in the oven had the most superior flavor, those stored in room temperature the second best flavor, and those stored in the refrigerator the least desirable flavor.

In a second trial beans treated according to the following precedure were used. The samples were killed by immersing them three times in hot water at 80° C. for 10 seconds at 30-second intervals. Following this the beans were kept in blankets for 1 day and then oven-sweated in blankets at 45° until flexible. After drying at room temperature to 28.6 percent of the freight weight, the beans were conditioned in sealed cans using four different temperatures: 45°, 35°, 27°, and 13°. After conditioning for 6 months it was found that those conditioned at 45° had the strongest vanilla aroma accompanied by a sweet prunelike note. The beans conditioned at 35° were not so strong but were more suave. Those kept at 27° and 13° were comparatively poor. Apparently high-temperature conditioning brought out background fixative qualities not so noticeable in the ordinary cured material. It is also important to note that the beans at 45° were completely cured after about 3 months of conditioning.

Extracts were prepared from these samples and when they were filtered through paper to remove fine solids it was noted that the higher the temperature at which the vanilla was conditioned the more rapid was the filtration. This indicated that more fine solids were suspended in the extracts of the beans conditioned at lower temperatures.

Mr. Rosenbaum said concerning these samples conditioned at 13° C., "No. 1 has a typically Puerto Rican light, flowery bouquet that is somewhat insipid all the way down. It has only a light flavor body that owes much of its character to vanillin. The deeper tones are almost lacking." Of those conditioned at 27°, he said: "No. 2, not quite as sweet and flowery as No. 1, but possesses more body, and a slightly better rounded character. Both No. 1 and 2 are lighter in body and depth than No. 3 and 4." Of the third sample, conditioned at 35°, he said: "No. 3 has a rich, winey character backed by the lighter fruity and flowery fractions. Much heavier and better rounded than No. 1 and 2, having a different shading which is closer to No. 4 in type. Some Mexican character." And he described those conditioned at 45° as follows: "No 4 slightly leathery odor indicative of higher fat content. Similar in character to No. 3 with rich, sweet body, and yet a note of the Puerto Rican character. Apparently heavier in end bouquet than any of the others, with a rich Mexican character."

Mr. Rosenbaum summed up his conclusions for all four tests as follows: "No. 1 and 2 are inferior to No. 3 and 4, with No. 1 a shade less desirable than No. 2. No. 3 and 4 are closer together in character,

with No. 3 being my first choice as the best of the lot, but a second examination changed my choice to No. 4 as the best rounded because of its heavier end bouquet."

Vanillin contents were 2.07 percent for the beans conditioned at 45° C., 2.21 percent for those at 35°, 2.55 percent for those at 27°, and 2.73 for those at 13°. The corresponding moisture contents were 14.1, 18.3, 20.5, and 18.5. Organoleptic tests of the extracts in ice cream showed that the extract from beans conditioned at 45° was superior by high significance to the others. Likewise, the extract from beans conditioned at 35° was significantly superior to those from beans conditioned at room and at refrigerator temperatures. Of the latter two, the extract from room-temperature-conditioned beans scored higher than that from cold-conditioned beans, but the difference was not significant.

It is concluded that conditioning at 45° C. was best, but that the beans should not be too dry at the beginning of conditioning. If the beans were dried to one-third of their fresh weight and then conditioned in closed containers at 45°, the product would not be so dry and the conditioning process would be complete in about 3 months.

RECOMMENDED CURING PROCEDURE

On the basis of the experimental work of the authors, the following curing procedure for whole beans is recommended:

(1) As soon as possible after harvest, wipe the beans with a damp cloth and kill by immersing them three times for 10 seconds in hot water (80° C.) at 30-second intervals.

(2) Sweat in sun in blankets, or in a closed oven at 45° C. containing a pan of water.

(3) Dry at room temperature to one-third of the original fresh weight.

(4) Tie beans in bundles, wrap the bundles in heavy paper or seal in jars or cans, and condition at 45° C. for 2 to 3 months until the batch has dried to one-fourth of the original fresh weight.

(5) Remove cured product and allow to air for 2 days.

(6) Wrap and pack for shipment.

SUMMARY

The criteria by which cured vanilla is judged were considered. It is concluded that, since no purely objective methods are available, the best procedure is to apply a subjective method, the results of which can be treated statistically to yield a valid measure of goodness. An organoleptic test well suited for this purpose is described. The test is made with ice cream containing the vanilla extract.

The effect of several killing methods on quality of the cured product was determined. The hot-water kill gave the best product, with freezing second, and scratching third. However, the latter had the highest vanillin content.

Beans cured whole, cut, and ground yielded extracts that were not greatly different in flavoring properties. The cut beans had a prune-like aroma but yielded a satisfactory extract. The ground beans were highest in vanillin.

Curing the pod separate from the seed and placental tissue showed that the pod portion accounted for the main part of the flavor of the bean.

Vanilla conditioned at 45° C. was superior to that conditioned at 35° which, in turn, was superior to the product stored at 27° or 13°.

These experiments show that vanilla-curing methods may be modified in two important respects: (1) Beans may be cut or ground before curing, thus simplifying the handling processes, and (2) conditioning may be carried out at 33°–45° C. to yield a superior product.

INACTIVATION AND VACUUM INFILTRATION OF VANILLA ENZYME SYSTEMS [1]

By MERRIAM A. JONES and GILDA C. VICENTE,[2] *chemist and collaborating chemist, respectively, Federal Experiment station, Office of Experiment Stations, United States Department of Agriculture, Mayagüez, P. R.*

INTRODUCTION

The term "curing" is commonly used to denote the over-all changes that take place during the processing of many different types of raw material to a finished product ready for the market. The term covers processing of such diverse products as vanilla, tobacco, cheese whisky, and derris root. The actual changes that occur in all of these curing processes may be basically different but they can be classified as: (1) Those involving the loss of water but no chemical transformations; and (2) those involving principally changes in the chemical composition and in which the loss of water is of only minor importance.

The first class, including such processes as the curing of derris root and of ginger tubers, mainly involves drying; any chemical changes that do occur may be deleterious. For example, under poor drying conditions, derris root curing results in browning and loss of rotenone.[3] Similarly, slowly dried ginger becomes discolored, hard, and of poor quality. The second class of curing, in which hydrolytic and oxidative changes are of more importance than the mere loss of water, is exemplified in such items as cheese and vanilla. This class can be further subdivided into two curing processes, in which the chemical changes are brought about principally by: (1) Enzymes, as represented by the hydrolytic cleavage of glucovanillin in vanilla, and (2) chemical transformation without enzymes, such as occurs in whisky curing. Those processes depending upon enzymatic action can be further classified according to the source of the enzyme into: Foreign enzymes such as those furnished by micro-organisms in the curing of cheese; and intracellular enzymes, which are contained in the material to be cured. These differences in curing processes are shown schematically in figure 1.

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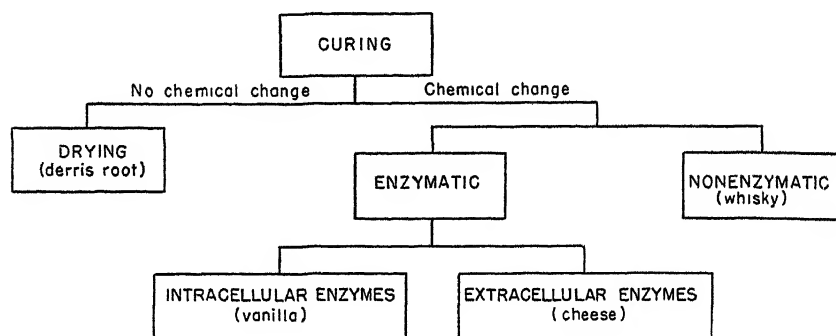
FIGURE 1.—Schematic arrangement to show the differences among curing processes.

THE VANILLA ENZYME SYSTEM

Vanilla curing is a process involving enzymes that are present in the tissue of the beans. An important point of inquiry is the part played by various types of enzymes in the curing process. It is well known that glucovanillin is hydrolyzed by a glucosidase and that there is some peroxidase activity in the beans. It was also found that excessive killing by prolonged immersion in hot water inhibited the browning system of the bean. In an unpublished report, E. K. Nelson, working at this station, stated that expressed juice of vanilla beans did not turn brown if kept from contact with air.

There are, then, two oxidizing enzymes in the whole tissue of the vanilla bean. One of these, a peroxidase, uses hydrogen peroxide. The other, a type of oxidase or aerobic dehydrogenase, utilizes molecular oxygen as acceptor for hydrogen removed from phenols that are being oxidized to stable pigments.

To study the oxidizing enzymes, a modification of the method of Miller and Dawson [4] was used. Briefly the procedure for testing by this technique is as follows: A known amount of enzyme is added to a buffered solution of catechol and a small amount of ascorbic acid in a flask mounted in a water bath. Air is bubbled through the solution and a capillary siphon is used to conduct the solution dropwise into a dish containing excess strong sulfuric acid, pyrogallol, potassium iodide, and starch solution.

The enzyme catalyzes the oxidation of catechol to a quinone which is immediately reduced again by the ascorbic acid. When all of the ascorbic acid is oxidized, free quinone siphons into the receiving dish and oxidizes the iodide ion to iodine which, in turn, yields a blue color with the starch. The acid is present to kill the enzyme coming from the siphon and the pyrogallol to inhibit premature oxidation of the iodide ion by air. The time required for development of the blue color is an inverse function of the activity of the enzyme.

For the work with vanilla beans the crude enzyme was not isolated, but a weighed amount of bean was crushed with sand in a mortar and

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[4] MILLER, W. H. and DAWSON, C. R. A NEW METHOD FOR THE MEASUREMENT OF TYOSINASE CATECHOLASE ACTIVITY. Amer. Chem. Soc. Jour. 63: 3375–3382. 1943.

added to the buffered catechol. Since the activity was low no ascorbic acid was added to lengthen the time for the test. The capillary siphon was equipped with a cotton filter to avoid stoppage by tissue fragments. For reaction times in excess of a few minutes the potassium iodide and starch were not mixed with the acid in the receiving dish because, in spite of the pyrogallol, the mixture would take on a purple hue. The siphon was placed above the dish containing the acid, and samples were removed periodically for test with potassium iodide and starch on a spot plate. Pyrogallol was omitted from the reaction. When the reaction time was expected to be long, the siphon was run only at intervals. The technique was tested with excellent results, using potato peel, which is known to contain an oxidase. For the record of results, an arbitrary scale was defined as follows: Positive results in 0 to 3 minutes, high activity; in 3 to 15 minutes, medium activity; in 15 to 30 minutes, low; and over 30 minutes, inactive.

In order to determine whether any ascorbic acid was present in the plant material that would retard the test for oxidizing enzymes, qualitative tests were made by adding methylene blue to a suspension of bean crushed in dilute oxalic acid. No bleaching took place when the blue suspension was exposed to sunlight. The addition of a minute crystal of ascorbic acid to the suspension caused rapid bleaching by sunlight. It can, therefore, be concluded that no appreciable amount of ascorbic acid was present in the bean tissue. The same results were obtained with slices of green and killed beans, but with cured beans the methylene blue was bleached, perhaps by some compound other than ascorbic acid. However, since cured beans gave positive results for oxidizing enzymes, this compound did not appear to interfere with the test.

When green vanilla beans were tested by the above method for oxidase activity, it was found that no color change resulted even after several hours. Of many samples run, only one, otherwise indistinguishable from the others, showed low activity. Sections of large blossom-end-yellow beans showed medium activity in the blossom end, but the middle section of the same bean was inactive. The yellow blossom end of small beans also tested negative.

Crushed green beans killed by immersing in a tube held in water at 65° C. for 3 minutes tested inactive after 1 day. However, beans with yellow and brown blossom ends, killed by freezing, showed high activity after 1 day. With these beans positive results were obtained when the catechol was omitted from the reaction flask, because the enzyme was sufficiently active to act upon the substrate present in the tissue. The tissue of the brown section showed high activity and that of the blossom-end-yellow section, medium activity.

Using bean tissue that showed no activity without the presence of catechol, some tests were made for specificity of the oxidizing enzyme. When tyrosine was substituted for catechol no action resulted. The contents of the flask took on a reddish cast. The addition of tyrosine to a suspension of crushed bean gave a black color after 1 day, indicating that vanilla has some tyrosinase activity. With a mixture of *o*-, *m*-, and *p*-cresol in place of catechol the activity of vanilla enzymes was medium. When these enzymes acted upon added vanillin as the substrate the activity was again medium.

To determine whether molecular oxygen was the acceptor in the reaction, the test was run on deaerated beans. A flask containing several beans was repeatedly evacuated and filled with nitrogen about every 5 minutes for several hours, after which the beans were crushed with sand in a mortar in a box while nitrogen was streamed into the mortar. The bean tissue was transferred to the reaction flask containing the solution previously boiled and then cooled in a nitrogen atmosphere. When the flask had been mounted, nitrogen instead of air was bubbled through and tests were made. After 30 minutes without results 50 mg. of vanillin were added. At the end of an additional 20 minutes without results, air was bubbled through, which produced positive results in 5 minutes. Upon repetition similar results were obtained. It is clear that molecular oxygen is necessary for the main enzymatic formation of quinone bodies in vanilla.

For further characterization of the enzyme the effect of inactivators was studied. It was found that the reaction occurred after 72 minutes in 0.001 M potassium cyanide. It was therefore reversibly inhibited by cyanide. Passing carbon monoxide at the rate of 4 cc. per second with air inhibited the reaction for several hours and developed a dark orange color similar to that brought about by the formation of a carbon monoxide-ferrous cysteine complex. Mercuric acetate, 0.001 M, also inhibited the reaction. When hydrogen sulfide was bubbled in with the air for 50 minutes, the reaction was inhibited; passing air through for 15 minutes after the hydrogen sulfide was shut off resulted in reaction. Whether the hydrogen sulfide reversibly inactivated the enzyme or whether it merely reduced quinones as fast as they were formed is not known.

Copper sulfate and ferric phosphate were tried as activators. Both gave a more rapid reaction but the results with copper were vitiated by the fact that the copper sulfate alone liberated the iodine. The iron salt, on the other hand, did not oxidize the iodide in the receiver but was capable of oxidizing catechol and vanillin in the aerated flask in the absence of plant tissue.

Tests for cytochrome oxidase by the "Nadi" reaction with p-diphenylenediamine and alpha-naphthol were positive for green beans both fresh and after killing in hot water and for brown beans killed by freezing. However, the test was negative for the latter type that had stood for 2 weeks.

The presence of catalase in green beans was shown by the decrease of hydrogen peroxide brought about by the addition of crushed beans to a buffered solution of hydrogen peroxide at $0°$ C., after the method given by Sumner and Somers.[5]

It appears from the foregoing observations that the oxidizing enzymes in vanilla form a complex system capable of oxidizing a variety of substrates including vanillin itself to quinone bodies and thence to condensed stable pigments. On the basis of the sensitivity to cyanide, the enzyme system appears to contain a heavy metal. Since it requires oxygen as an acceptor and is inactivated by carbon monoxide, the main action of oxidation seems to be carried on by an

[5] SUMNER, J. B., and SOMERS, G. F. CHEMISTRY AND METHODS OF ENZYMES. 365 pp., illus. New York. 1943. (See p. 171.)

oxidase rather than a peroxidase. The manner in which this system should be controlled in order to obtain an optimum cure of vanilla beans remains to be investigated.

EFFECT OF INACTIVATION OF THE ENZYMES

Curing may be defined as indicated in the introduction. However, for the purpose of clarifying thought, it should be stated that many curing processes are not entirely of one type or another. For example, vanilla curing, commonly considered to depend upon enzymes, may, at least in certain phases, be nonenzymatic. It is clear that during the early stages of curing, enzymatic oxidation occurs and it has long been known that the cleavage of glucovanillin to glucose and vanillin is brought about by a hydrolytic enzyme. The importance of enzymes in the later stages of curing was not considered.

With this in mind, therefore, beans were autoclaved at various stages of the curing process to determine the quality of the resulting product. Autoclaving at 120° C. was calculated to destroy the enzyme system completely so that subsequent changes could be considered nonenzymatic in nature. However, it was found that although oxidase, catalase, and peroxidase activities were destroyed by autoclaving, a peroxidase activity later appeared again, at least to a certain extent. For this experiment, triplicate 250-gm. samples of vanilla beans 6 to 8 inches long, with about $\frac{1}{2}$ inch of yellow at the apical end, were killed by immersing three times for 10 seconds at 30-second intervals in hot water (80°). They were then sweated at 45° until flexible and dried at room temperature to 28.6 percent of the fresh weight. The samples were conditioned at room temperature in closed boxes. Groups of samples were autoclaved at the following stages: Immediately after killing, 2 days after killing, at the end of the sweating, and at the beginning of conditioning. At the beginning of the experiment, fresh weights and the moisture content were determined and during the experiment, data on weight, color, aroma, and mold were obtained. At each stage the activities of oxidase, catalase, and peroxidase were measured.

The controls became dark brown and oily after killing. During drying they became more sticky and developed some vanilla aroma. During conditioning the stickiness subsided and the vanilla aroma became more developed. Before killing some catalase activity was found, but after killing none could be shown. Likewise, oxidase, although it is known to be important, was not evident after killing. A high peroxidase activity was noted in the fresh beans. If the value of peroxidase activity of fresh beans is taken as 100 percent, the activity in the controls rose to 115 after killing; 2 days later, 133; dropped to 94 after sweating, and rose to 270 at the beginning of conditioning. After about 2 months' conditioning the activity was 400 percent of that of the fresh beans.

The samples autoclaved after killing stayed green for several weeks and finally became greenish yellow. They were oily, flaccid, and dried the most rapidly of all the samples. During drying the sticky resin became harder and the beans took on a burnt fermented aroma unlike the aroma of vanilla. The same properties were noted during con-

ditioning except that the oiliness subsided and mold developed. No vanilla aroma could be detected. The peroxidase activity dropped to 19 percent and was 20 at the beginning of conditioning.

The beans autoclaved 2 days after killing were light brown, oily, and less flaccid in texture and less disagreeable in aroma than those autoclaved immediately after killing. Upon drying, the resinous oil hardened and some mold developed but was eradicated.[6] After conditioning, the beans were neither oily nor sticky and did not develop a vanilla aroma. The peroxidase activity dropped from 133 to 16 after autoclaving and then recovered to 36 at 12 days, 29 at 43 days, and after 3 months was again 16.

The samples autoclaved 12 days after killing were more like the controls throughout the curing except that the vanilla aroma was not so well developed and the color was lighter brown. The peroxidase activity dropped to 9 percent of the original value upon autoclaving and then recovered to 16 at 43 days, and 12 percent at 3 months.

The beans autoclaved 1 month after killing were similar in quality to the controls; they became dark brown, were not flaccid, and developed a vanilla aroma which was as strong as that of the controls. The peroxidase activity dropped to 23 after autclaving and to 13 after 2 months' conditioning.

Analyses showed that the controls contained 2.44 percent of vanillin, dry basis. Those autoclaved immediately after killing contained 0.31 percent; 2 days after killing, 0.46 percent; 12 days after killing (the end of the sweating process), 1.54 percent; and 42 days after killing (beginning of conditioning), 1.99 percent. The extract of the beans autoclaved immediately after killing was light brown and, as the time of autoclaving after killing was increased, the color was darker. It was concluded that the main part of the hydrolysis of glucovanillin occurred during the first 2 weeks but that some hydrolysis took place during the early part of conditioning. Half of the glucovanillin was hydrolyzed during the first 9 days after killing.

From the results obtained it appears that the principal changes brought about by enzymes occurred during the first 2 weeks of curing. Enzymatic transformations are important during killing and gradually become less so until, by the end of the sweating period, enzymatic changes are unimportant. The fact that peroxidase, after being inactivated by autoclaving, appeared to recover to about the same extent in all of the treatments, indicates that this activity is probably not responsible for changes subsequent to sweating. This is also indicated by the fact that, although the peroxidase activity was exceedingly high in the controls and comparatively low in those autoclaved 12 days and 43 days after killing, the vanilla characteristics were about the same in all cases.

Presumably, then, since the enzymatic changes take place during the first few days of curing and since the principal development of vanilla aroma occurs during the conditioning, a considerable part of the curing process must be nonenzymatic.

It was also found that oxidase, as well as peroxidase, "came back."

[6] "Mycoban" (sodium propionate), either in saturated alcoholic solution or as a powder, has been found to be rather effective in eradicating mold on vanilla.

This was shown by autoclaving two samples of cut beans, after which one was kept sealed while the other was exposed to air but not enough to allow it to dry.   After 2 weeks the pieces exposed to air began to brown but those sealed remained green.   After 6 months the exposed sample had a weak, sweetish aroma, not like vanilla, while the sealed sample was still green.   Therefore, although recovery of oxidase could not be shown by a direct measure of oxidase activity, the foregoing observations showed that an apparent "rejuvenation" occurred.

In the light of recent knowledge concerning nonenzymatic browning (Maillard reaction) the foregoing observations might be otherwise interpreted.   The apparent rejuvenation was probably illusory in that the chemical reactions which indicated the activity of the enzymes may have occurred because of the chemical reactivity of products formed during the nonenzymatic browning reactions.   Viewed in this manner, it may be said that no reactivation of enzymes occurred and further that, while the hydrolysis of glucovanillin and part of the coloration were due to enzymatic changes, a considerable part of the flavor development was due to nonenzymatic processes.   This experiment then suggests a starting point for further study of the relation between flavor development and the browning reaction.

VACUUM INFILTRATION OF ENZYMES

It is well known that an enzymatic step is involved in the hydrolysis of glucovanillin and in some of the oxidative changes that result in browning.   An experiment was conducted, therefore, to learn more about the general nature of the enzyme system that acts in the curing process.   Cut beans were autoclaved to inactivate the natural enzymes and were then infiltrated with crude enzyme extracts of materials whose enzymatic character is known to some extent.   The products were then cured and the results noted.

For this purpose 150-gm. samples of blossom-end-yellow vanilla beans cut to 5–mm. slices were autoclaved for 5 minutes at 120° C. An untreated sample was also prepared which was killed by heating in an oven at 60° for 1 day and then sweated until the pieces lost turgidity and became brown.   After drying at room temperature to 28.6 percent of the original weight, the control was conditioned in a closed jar. Immediately after autoclaving and cooling the samples to be treated were covered with an extract of the enzyme to be infiltrated and placed in a vacuum chamber.   Upon evacuation to 4 cm. pressure, the air in the cells of the plant tissue bubbled out.   The vacuum was released about 5 minutes after bubbling ceased and, as air entered the chamber, the cells filled up with the extract in which the pieces were submerged. After a half hour the infiltrated pieces were drained and cured in the same manner as the control.

The enzyme extracts were prepared as follows: One-half percent yeast extract was made by mixing brewer's yeast with water in a Waring Blendor for 10 minutes.   The suspension was then centrifuged for 10 minutes, the precipitate rejected, and the clear liquid containing the enzyme was used.   A ½-percent solution of emulsin was prepared in the same manner.

To make the oxidase solution 1 pound of frozen mushroom, *Agaricus campestris*, was passed through a food chopper into 2 volumes of cold acetone chilled with dry ice to precipitate the enzyme and wash out the natural substrate and coloring matter.[7] It was filtered immediately and the pulp frozen for 2 hours with dry ice after which it was broken up with 600 cc. of water containing a drop of concentrated ammonium hydroxide. The filtrate from this contained the enzyme.

Vanilla enzyme was prepared in the same manner, using vanilla beans instead of mushroom.

The peroxidase extract was prepared by grinding 1 pound of frozen turnips several times through a food chopper and then squeezing the juice through cheesecloth. One gram of purified diatomaceous earth was added for each 100 cc. of juice and the suspension filtered after 10 minutes through paper on a Buchner funnel containing a layer of filter aid. The liquid which contained the enzyme was clear yellow.

The enzyme activities of the preparation are given in table 1.

TABLE 1.—*Activities of enzyme preparations used for vacuum infiltration of vanilla*

Source	Peroxidase	Oxidase
Vanilla	Medium	High.
Yeast	None	None.
Emulsin	____do	Do.
Mushroom	Low	High.
Turnip	High	None.

The vanilla samples were infiltrated with extracts representing a like amount of the other plant materials. This experiment was carried out twice because, in the first run, oxidation took place in the autoclaved samples and considerable mold appeared. However, the results were similar in both trials. The results are summarized in table 2.

It is clear from the results that, after the natural vanilla enzymes were inactivated, infiltration with an oxidase preparation gave a product that resembled the ordinary cured vanilla more closely than did the products obtained by using other enzyme preparations. In samples treated with oxidase diluted with other enzymes the product was poorer. This experiment further confirms the conclusion that an oxidase is responsible for most of the enzymatic oxidizing action in the development of vanilla aroma that occurs during curing.

SUMMARY

The nature of the process of vanilla curing is discussed.

The oxidase system in vanilla was briefly investigated.

The activities of the oxidase, peroxidase, and catalase during curing were measured and it was concluded that the main enzymatic change is due to an oxidase system.

[7] MILLER, W. H., MALLETTE, M. F., LLOYD, J. R., and DAWSON, C. R. A NEW METHOD FOR THE MEASUREMENT OF TYROSINASE CATECHOLASE ACTIVITY. II.—CATECHOLASE ACTIVITY BASED ON THE INITIAL REACTION VELOCITY. Amer. Chem. Soc. Jour. 66: 514–519. 1944.

TABLE 2.—*Results of vacuum infiltration of autoclaved vanilla beans with some crude enzyme extracts of other materials*

Enzyme		Result		
Source	Nature	Color	Aroma	Remarks
Control____	_____	Dark brown_ _____	Prunelike vanilla_____	Good vanilla.
Control [1]__	_____	Greenish brown_____	Weak acid_____	No cure.
Vanilla_____	Glucosidase, oxidase, peroxidase.	Dark brown and dark green.	Weak acid, some sweet note, no vanilla.	Moldy.
Water_____	_____	Greenish brown_____	Weak acid_____	No cure.
Yeast_____	Alpha-glucosidase___	Dark brown_ _____	Acid, no vanilla_____	Moldy.
Emulsin___	Beta-glucosidase____	Dark brown outside and yellow inside.	Acid, no vanilla_____	Poor.
Mushroom.	Oxidase_____	Dark brown_____	Prunelike vanilla_____	Weaker than control.
Turnip_____	Peroxidase_____	Dark brown_____	Acid_____	Poor.
Mixture____	Oxidase-peroxidase__	Light to dark brown__	Acid, slight vanilla____	Not so good as control.
Mixture____	Emulsin-oxidase____	Light to dark brown__	Prunelike vanilla_____	Weaker than control.
Mixture____	Emulsin-peroxidase_	Dark brown_____	Acid_____	Poor.
Mixture____	Emulsin-peroxidase-oxidase.	Light to dark brown__	Prunelike, slightly acid.	Not so good as control.
Mixture____	Yeast-emulsin_____	Light to dark brown__	Acid, slightly sweet, no vanilla.	Poor.
Mixture____	Yeast-oxidase_____	Light to dark brown__	Prunelike vanilla_____	Weaker than control.
Mixture____	Yeast-peroxidase_ ___	Light to dark brown__	Acid_____	Poor.
Mixture____	Yeast-oxidase-peroxidase.	Light to dark brown__	Acid_____	Poor.
Mixture____	Yeast, emulsin, oxidase, peroxidase.	Light to dark brown__	Acid_____	Poor.

[1] Autoclaved but not infiltrated.

It was found that the main enzymatic changes occur during the first 12 days of curing but the main development of flavor occurred after this time. A considerable part of the curing process appears to be nonenzymatic.

Vanilla with inactivated enzyme system was infiltrated with enzyme extracts of several types to find that an oxidase-type extract gave a product most closely resembling vanilla.

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QUALITY OF CURED VANILLA IN RELATION TO SOME NATURAL FACTORS [1]

By MERRIAM A. JONES and GILDA C. VICENTE,[2] *chemist and collaborating chemist, respectively, Federal Experimental Station, Office of Experiment Stations, United States Department of Agriculture, Mayagüez, P. R.*

INTRODUCTION

During the vanilla-curing season of 1945–46 studies were made for the purpose of correlating the quality of cured vanilla with several natural factors which, by their effects on the developing bean, might affect the quality of the cured product. For this work a standard curing and testing procedure was used so that the quality of the cured beans would depend only on the conditions under which they were grown and harvested.

VARIATION OF QUALITY DURING SEASON

The fruit of *Vanilla fragrans* (Salisb.) Ames reaches its full size several months before it is ready for harvest. The time required for the full-sized pod to ripen for harvest varies to the extent that in Puerto Rico some pods are ready for harvest early in November, most are ready in late December and early January, while some are not ripe until February. The time at which the peak of the harvest season occurs varies somewhat from year to year, depending on climatic conditions. Also it is notable that the early beans are usually smaller than those that ripen later. Since it was considered probable that the time of the season at which the pods ripen might affect the quality of the cured product, some trials were made in which beans were harvested early in the season, at the peak of the season, and in late season. Beans were collected on November 3, December 27, and January 31. To insure a uniform degree of maturity, all beans were harvested when the blossom end was yellow. The location of the vanillery was near Mayaguez on Las Mesas at an elevation of 600 feet.

Soon after harvest the pods were wiped with a damp cloth and killed by immersing in hot water (80° C.) three times for 10 seconds at 30-second intervals. This was followed by sweating in an electric oven at 45° until the beans were flexible. They were then dried on drying racks at room temperature to one-third of fresh weight. Conditioning was then carried out by storing in closed vessels at 45° for 2 months by which time the weight was one-fourth of the fresh weight. No mold developed on any of the samples.

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[1] Received for publication January 3, 1949.

[2] "Criteria for Testing Vanilla in Relation to Killing and Curing Methods," and "Inactivation and Vacuum Infiltration of Vanilla Enzyme Systems," are parts of this same series and are published in the Journal of Agricultural Research, vol. 78, no. 11.

The beans harvested early in the season were 4½ to 7½ inches long with most of them about 5½. The moisture content was 80.07 percent. During sweating and drying a strong vanilla aroma developed which was considered very good. After the beans had been conditioned the aroma was even stronger and the beans were somewhat oily and black. In general, the cured product resembled Mexican vanilla. No vanillin crystallized on the surface of the beans.

The beans harvested at the height of the season were 8 to 9½ inches long and averaged 74.48 percent moisture. During sweating and drying the product developed a slight vanilla aroma accompanied by a sweetish note. The aroma lacked strength and was definitely inferior in quality to that of beans harvested early in the season. During conditioning the aroma improved but still lacked strength. The beans were shiny and oily which gave them a pleasing appearance although they were somewhat light in color. No vanillin crystallized on the beans.

The beans harvested late in the season were 8 to 10 inches long and averaged 79.66 percent moisture. During the sweating and drying the samples developed a bouquet similar to that of the beans harvested at midseason. The aroma improved during conditioning. A considerable amount of vanillin crystallized on the surface of the beans, which improved the appearance of the product, but otherwise there was little difference in aroma and appearance of these beans and those harvested at midseason.

The moisture and vanillin contents of the cured products are tabulated in table 1.

TABLE 1.—*Moisture and vanillin contents of cured vanilla harvested at different times of the harvesting season*

Harvesttime	Moisture	Vanillin [1] [2]
	Percent	*Percent*
Early, Nov. 3	21.37	2.67
Middle, Dec. 27	27.61	3.59
Late, Jan. 31	26.82	4.17

[1] The vanillin content was calculated on the dry basis.
[2] Vanillin analyses were made according to the A. O. A. C. method. ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS. OFFICIAL AND TENTATIVE METHODS OF ANALYSIS. . . . Ed. 5, 757 pp., illus. Washington, D. C. 1940. (Pp. 320–321.)

Extracts representing equal amounts of dry vanilla were prepared [3] and compared by a method recommended by Gnadinger.[4] This method consisted of diluting the extract with 19 parts of water to 1 part of extract. According to this test beans harvested early in the season had a stronger vanilla flavor than beans harvested later in the season. Concoctions containing 1 cc. of extract in 50 cc. of milk were also tested with the same results.

[3] The method used for the preparation of extracts was outlined in the first paper of this series, published in the Journal of Agricultural Research, vol. 78, no. 11.
[4] GNADINGER, C. B. VANILLA. 60 pp. Minneapolis, Minn. 1929.

From these results it appears that beans harvested early in the season, although smaller and lower in vanillin content, cure to a superior product as judged by aroma and flavor.

EFFECT OF MATURITY OF VANILLA ON QUALITY OF CURED PRODUCT

In previous work at this station it was shown that beans harvested when the blossom end had turned yellow gave a cured product superior in aroma and in vanillin content to that from beans harvested entirely green. In other work beans harvested after they had turned brown on the vine were shown to give a cured product higher in vanillin than the product from green beans but lower than that from blossom-end-yellow beans. Because of these observations it is recommended that vanilla be harvested when the blossom end is light yellow and before splitting begins. Split beans are discounted on the market although some claim that the split product yields a superior extract. The work was repeated with beans picked at several stages of maturity: (1) Entirely green, (2) blossom end yellow, (3) blossom end brown, and (4) entirely chocolate. The beans used were grown in a vanillery on the station grounds.

During sweating the beans harvested green developed a slightly fermented aroma which disapeared during drying. Vanilla aroma was faint but improved during conditioning and was acompanied by a sweetish note. However, the vanilla aroma did not become so strong or as true as that of a high-quality cure. The beans were dull light brown with a gummy texture. It was clear that vanilla should not be harvested at this stage of maturity.

The samples harvested with the blossom end yellow gave a good cured product, dark brown, oily, and with a pleasing vanilla bouquet.

Those harvested with the blossom end brown developed a fruity aroma which was agreeable but lacking in true vanilla character. Later the aroma became fragrant and similar to that of beans killed by scratching. Most of the beans were split. Much vanillin crystallized on the surface of these beans.

Beans harvested when brown cured to a product with an aroma similar to but weaker than that of the beans harvested with the blossom end brown. There was profuse vanillin crystallization on these beans.

Extracts were prepared and vanillin analyses made. The data are given in table 2.

TABLE 2.—*Analyses of vanilla beans harvested at several stages of maturity*

Stage of maturity when harvested	Moisture content		Vanillin, dry basis
	Fresh	Cured	
	Percent	*Percent*	*Percent*
Green	82.14	33.92	2.86
Blossom end yellow	80.47	24.12	3.50
Blossom end brown	67.05	22.76	3.79
Brown	59.07	12.62	3.40

From the figures presented in the table it appears that the best vanilla is obtained from beans harvested when the blossom end is yellow. This corroborates data obtained in previous experiments.

ALTITUDE OF PLANTING AS A FACTOR IN QUALITY OF CURED VANILLA

Among the many possible factors that might affect the quality of the cured product is the altitude at which the vanillery is located. Temperature apparently has something to do with the time of bean set; hence, it is possible that an altitude effect due to temperature difference could determine to some extent the quality of cured beans. In a preliminary effort to measure the effect of altitude on the quality of cured vanilla, an experiment was made in which vanilla beans were harvested from vanilleries at several altitudes. No final evaluation is possible at this time because in this experiment there were several other differences among the samples beside those due to altitude alone.

Beans were harvested when the blossom end was yellow and cured by the process outlined previously. Samples were obtained from the following locations and altitudes: Mayaguez, 80 feet altitude; Las Mesas, 600 feet; Morovis, 700 feet; and Castañer, 1,800 feet.

During the sweating phase of the curing, beans from Mayaguez developed a strong vanilla aroma which persisted throughout the process. The product was oily, shiny, dark in color, and possessed a very good aroma and appearance.

The samples from Las Mesas, although they appeared to be the best beans when fresh, did not develop a full vanilla aroma upon curing. The character of the aroma was faint and had a sweetish note. The beans were somewhat oily and lighter in final color than those from Mayaguez.

Beans harvested at Morovis developed an appearance and aroma characteristic of beans harvested entirely green. They were dull, light brown in color, not oily, and gummy in texture. Only a slight vanilla aroma developed.

The Castañer product developed a suave vanilla aroma with a sweetish note during the sweating. There was also a foreign, somewhat flowery odor present. After conditioning, the beans had a good vanilla aroma which was not so strong as that of the beans from Mayaguez. No vanillin crystallized on the surface of any of the samples. The data on beans from different altitudes are presented in table 3.

TABLE 3.—*Analyses of vanilla beans harvested at several altitudes in Puerto Rico*

Location	Elevation	Moisture content		Vanillin content, dry basis
		Fresh	Cured	
	Feet	*Percent*	*Percent*	*Percent*
Mayaguez	80	77.07	25.50	3.79
Las Mesas	600	81.26	36.06	3.45
Morovis	700	81.58	21.97	2.96
Castañer	1,800	78.82	30.85	3.75

Extracts prepared from these beams were tested in milk and in water and ranked as follows in decreasing order of quality: Mayaguez, Castañer, Las Mesas, Morovis.

From this experiment it appears that any effect that the altitude at which the plant grows may have on vanilla quality was masked by other factors such as soil, mulch, or rainfall distribution.

QUALITY OF VANILLA FROM DISEASED PLANTS

Another factor that might be expected to determine, at least to some extent, the quality of vanilla beans is the state of health of the plant. It is known that the quality of many fruits is influenced by the vigor of the plant. Although vanilla root rot is a localized disease, it causes general wilting of the plant and ultimate death. Therefore, it was considered possible that beans from plants with root rot might differ in the quality of the cured product from those grown on healthy plants.

To test this hypothesis duplicate samples of beans were collected from 16 plants, 8 of which were healthy and 8 of which were suffering from root rot. The beans from healthy plants were 7½ to 9 inches long. The samples cured by the procedure outlined in the first section appeared to be similar, and no differences in aromatic qualities could be detected. Both lots had good vanilla character. There was no vanillin crystallization or mold development on any of the samples. The analytical data are given in table 4.

TABLE 4.—*Analyses of vanilla beans from healthy and from diseased plants*

State of health of plant	Moisture content		Vanillin content, dry basis
	Fresh	Cured	
	Percent	*Percent*	*Percent*
Healthy	78.64	33.38	3 45
Diseased	80.22	36.56	2.97

Extracts of these samples were tested organoleptically in water and in milk with the result that no differences in flavoring properties could be established. From these results it can be concluded that, although the effect of vanilla root rot is to decrease the quantity of beans by weakening the plant, the disease does not affect the quality of the cured product.

SUMMARY

Some experiments were made for the purpose of relating the quality of cured vanilla with the conditions under which the plant was grown.

It was found that vanilla beans that ripened early in the harvesting season, although smaller than those that ripened later, gave a cured product of somewhat higher quality than those harvested at mid-season or late in the season.

Beans were harvested at several stages of maturity and it was found that green beans cured to an inferior product. The best cured vanilla was obtained from beans harvested when the blossom end of the fruit was yellow.

As far as could be determined, the altitude of the vanillery at which the beans were harvested did not have any effect on the quality of the cured beans.   If any differences due solely to altitude were present, they were masked by other factors.

Beans from diseased plants were about the same in final quality as those from healthy plants, but those from diseased plants were some-what smaller.

RATE OF DEVELOPMENT OF CALIFORNIA RED SCALES RESISTANT AND NONRESISTANT TO HYDROCYANIC ACID GAS, AS AFFECTED BY TEMPERATURE [1]

By Francis Munger [2]

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INTRODUCTION

The rate of development of the California red scale (*Aonidiella aurantii* (Mask.)) in California has been discussed by Quayle [3] and by Bliss, Broadbent, and Watson.[4] Their work was done before the genetic basis of differences in resistance to hydrocyanic acid gas had been established by Dickson [5] and Yust et al.[6] In previous work the writer [7] found no differences in reproduction and mortality between resistant and nonresistant scales. Studies on the rate of development of the same two strains were then made to determine whether there were any differences that might affect the relative growth rates of populations of the two strains in the field.

METHODS

The methods of handling and rearing the insects were similar to those described by Munger.[7] The periods measured were the first instar of both males and females and the second instar and the third stage of the females. The third stage included the period from completion of the second molt to production of the first young. The development of the males was not followed after they became recognizable as such in the second instar.

EXPERIMENTAL RESULTS

DEVELOPMENT AT CONSTANT TEMPERATURES

In the first experiment scales were subjected to a series of five constant temperatures—93.2°, 86.0°, 78.8°, 71.6, and 64.4° F. The extremes of temperature represented in this series were near the upper and lower limits at which the life cycle could be completed. Because of the slow rate of development at 64.4°, observations for all stages at that temperature were made at 24-hour intervals. Observations at the other temperatures were made at 12-hour intervals, except for the third stage, which were also made at 24-hour intervals.

Six lemons were used for tests at each temperature. All the lemons were infested by the half-lemon technique at 78.8° F. over periods ranging from 60 to 90 minutes. Immediately after being infested they were placed in constant-temperature cabinets. On three lemons of each group the resistant strain was on the upper half, and on the other three it was on the lower half. The mid-infesting time was used as the theoretical beginning of the test. During the first instar 60 scales on each half lemon were individually numbered with India ink. Inspections were started before any scales began to transform and were continued until the scales had either died or reproduced, or until the lemon had become hard or spoiled. Except for the time required to examine the scales, and for some minor irregularities in the control apparatus, the temperatures were held within ±0.9°. Continuous air circulation was provided by blowers or fans. All inspections were made at 78.8°, except for the scales subjected to 64.4°, which were examined at 59°–60.8°. Humidity was maintained in the chambers at approximately 68 percent for 64.4°, 56 percent for 71.6°, 60 percent for 78.8°, 44 percent for 86.0°, and 43 percent for 93.2°.

During the inspections the lemons were removed from the cabinet one at a time. The examination of the scales on each lemon required approximately 7.5 minutes. Radiant heat from the microscope lamp during the examination was nearly eliminated by passing the light through a glass vessel containing copper sulfate in water. The color and appearance of the scale cover was used as the criterion of development. In preliminary work it was found that the end of the first instar of both sexes is marked by a rather definite change in the appearance of the scale cover. The yellow color becomes more orange, and the appearance suggests dryness and opacity. This change is associated with the separation of the scale body from the cover. The end of the second instar in the female is marked by a similar change in appearance, but the scale is much larger and the outline of the body can usually be seen to have drawn away from the cover. The inspections were made under a magnification of 20.7.

The appearance of crawlers under the cover was used as an index of reproduction. The crawlers were usually, but not always, visible through the cover.

To check the uniformity of results in different generations, and to provide data at additional temperatures, a second constant-temperature experiment was carried out with a later generation of scales. Two new temperatures, 82.4° and 75.2° F., were used, and 78.8° was

repeated. The technique was similar to that used in the first experiment, except that inspections were made at 24-hour intervals and the third stage was not measured. Inspections were made at temperatures approximating those in the cabinet. The results of these experiments are summarized in table 1. The values shown are averages for all the insects.

TABLE 1.—*Hours required by red scales for completion of life stages at different constant temperatures*

FIRST EXPERIMENT

Temperature (°F.)	First instar				Second-instar female		Third-stage female		Complete development of females	
	Male		Female							
	Resistant	Nonresistant	Resistant	Nonresistant	Resistant	Nonresistant	Resistant	Nonresistant	Resistant	Nonresistant
93.2	176	189	182	179	278	262	569	550	1,006	980
86.0	186	181	194	189	251	246	444	441	885	873
78.8	220	221	239	228	269	270	495	491	1,002	989
71.6	344	349	361	360	443	449	831	838	1,614	1,621
64.4	604	610	622	631	787	790	1,497	....	2,837	....

SECOND EXPERIMENT

82.4	198	198	206	203	250	253	....	....	....	....
78.8	233	235	249	237	293	270	....	....	....	....
75.2	277	286	298	301	352	349	....	....	....	....

The data of the first experiment indicate that the nonresistant scales may grow a little more rapidly than the resistant scales at the higher temperatures and possibly slightly more slowly at the lower temperatures. However, the test at 86° F. was the only one showing a statistically significant difference ($P=0.01$) between the two strains in the time required to complete development. All the differences were very small.

The analysis of variance of the data for 78.8° F. (the only temperature common to the two experiments) showed a significant difference at the 1-percent level between the two experiments. At that temperature scales in the second experiment required from 9 to 14 hours longer to complete the first instar than did those in the first experiment. The resistant female scales required 24 more hours to complete the second instar in the second experiment, but there was no difference in the nonresistant strain. There was also a significant difference between the sexes in the first instar; except for the nonresistant scales at 93.2°, the first instar in the males was consistently shorter than in the females in both experiments.

Except where otherwise stated, 360 scales of each strain were tested at each temperature in each experiment. The fate of all the scales in the two experiments is shown in table 2.

TABLE 2.—*Number of red scales that attained various stages of development in constant-temperature experiments*

FIRST EXPERIMENT

Temperature (° F.)	Died in first instar		Died in second instar		Males in second instar		Died in third stage		Reproduced	
	Resist-ant	Nonre-sistant	Resist-ant	Nonre-sistant	Resist-ant	Nonre-sistant	Resist-ant	Nonre-sistant	Resist-ant	Nonre-sistant
64.4	37	31	184	178	45	53	82	98	12	0
71.6	83	67	60	56	78	95	70	68	69	74
78.8 [1]	121	99	10	6	113	118	8	6	108	129
86.0	34	32	27	17	126	148	27	16	146	147
93.2	39	15	52	35	95	123	153	135	21	52

SECOND EXPERIMENT

Temperature (° F.)	Died in first instar		Died in second instar		Males in second instar		Died in third stage		Reproduced	
75.2	50	70	85	73	99	103				
78.8	56	40	50	49	126	145				
82.4	42	50	55	55	117	95				

[1] Only 358 nonresistant scales were used at this temperature.

In the first experiment the mortality of the first-instar scales appeared rather high, but it was followed by a low mortality in the second instar. Between 64.4° and 86° F. the number of females that survived to reproduce increased with increasing temperatures. More of the resistant females produced young at 64.4° and more of the nonresistant reproduced at 93.2°; otherwise there was little difference in survival of the two strains. In the second experiment no large differences in mortality occurred between the strains. At 78.8° fewer scales died in the first instar than in the first experiment, but there was a correspondingly higher mortality in the second instar.

DEVELOPMENT AT FLUCTUATING TEMPERATURES

Two experiments were made to determine the relative rate of development of the two strains of the California red scale under fluctuating temperatures. The first experiment was designed to represent the general range of moderate spring and summer temperatures. The scales were exposed to temperatures that fluctuated uniformly between a minimum of 64.4° F. at 5 a. m. and a maximum of 86° at 5 p. m. each day. Inspections were made at 12-hour intervals for the first and second instars and at 24-hour intervals for the third-stage female.

The temperatures for the second experiment, representative of high summer temperatures which may occur in interior California, were selected from the thermograph records of Corona over a 7-week period from July 8 to August 26, 1935. The records for this period were transcribed to the reproducing element of a variable thermostat in order to recreate the same conditions in the laboratory. In this period 35 days had a minimum of less than 60° F. In 11 days the minima ranged from 47.5° to 50°. Twenty days had a maximum of more than 100°, and on 7 days the maximum temperature ranged from 105.1° to 107.8°. Inspections were made at 24-hour intervals. The second molt of the scales was completed, but no reproduction

occurred within the 7-week period. The first 4 weeks of the program were then repeated, after which the lemons were transferred to a constant temperature of 77° for the last 5 days of the experiment. During the developmental period of the scales the lemons were in good condition, but near the end of the experiment they were beginning to harden. In each program 360 scales of each strain were tested.

The results of these experiments are shown in table 3.

TABLE 3.—*Hours required for development of red scales in 2 fluctuating-temperature programs*

Stage and sex	64.4° to 86° F.		Corona high-temperature program	
	Resistant	Nonresistant	Resistant	Nonresistant
First-instar male	299.6	296.5	369.5	354.8
First-instar female	319.1	303.0	366.8	360.7
Second-instar female	361.6	349.3	455.4	456.6
Third-stage female	694.4	676.9	767.8	758.1
Complete development of females	1,366.0	1,321.1	1,588.5	1,570.7

In the experiment at moderate temperatures the resistant scales required about 45 hours more than the nonresistant scales to complete their development, a difference that was possibly significant ($P=0.05$). In the other experiment with a wider range of temperatures the difference between strains was not significant.

The fate of the scales in the experiments at fluctuating temperatures is shown in table 4.

TABLE 4.—*Number of scales that attained various stages of development in 2 fluctuating-temperature programs*

Fate of scales	64.4° to 86° F.		Corona high-temperature program	
	Resistant	Nonresistant	Resistant	Nonresistant
Died in first instar	55	63	39	37
Died in second instar	85	64	35	30
Distinguishable as males in second instar	97	114	115	122
Died in third stage without reproducing	22	14	21	24
Reproduced	101	105	150	147

There was no apparent trend in the mortality of the scales in the two experiments. The numbers that reproduced were nearly equal.

DISCUSSION

In these experiments the differences between strains, if any, were very small and were not conclusively demonstrated. The largest difference observed was about 3 percent of the total period. Such differences, if occurring consistently, would require a long period of time to have much biological effect on the population and seem unimportant relative to the selective action of hydrocyanic acid gas that has been demonstrated in fumigation of mixed populations.

The records on mortality in these experiments, as well as the more extensive data reported earlier,[8] have not shown any consistent differences between strains.

SUMMARY

The rate of development of strains of the California red scale (*Aonidiella aurantii* (Mask.)) resistant and nonresistant to hydrocyanic acid gas has been studied under constant temperatures ranging from 64.4° to 93.2° F. and under two fluctuating-temperature programs. One fluctuating-temperature program, representing moderate spring and summer conditions, ranged from 64.4° to 86°; the other, representing hot interior California conditions, ranged from 47.5° to 107.8°. In two of the tests (64.4° to 86° fluctuating and 86° constant) small differences were detected between the strains. There were no statistically significant differences at other temperatures, but there was a suggestion that a slight difference may exist between the strains. The resistant scales may develop less rapidly than the nonresistant scales at high temperatures and perhaps more rapidly than the latter at low temperatures.

In the constant-temperature experiment a small but significant difference in the rate of development of scales of two generations was found.

In the range of temperature from 64.4° to 93.2° F. the number of females that survived to reproduce increased with increasing temperatures. A larger number of resistant than nonresistant females produced young at 64.4° and larger number of the nonresistant females reproduced at 93.2°.

In the fluctuating-temperature experiments there was no apparent difference in mortality between the strains of scales. In the two experiments the numbers of scales that lived to reproduce were nearly equal.

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[8] See footnote 7, p. 451.

GROWTH OF FIRST-GENERATION CROSSBRED DAIRY CALVES [1]

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INTRODUCTION

One of the chief questions of interest in all cross-breeding investigations is whether or not the crossbred progeny will exhibit any evidence of heterosis, or so-called "hybrid vigor." In view of the fact that heterosis, as indicated by the increased rate of growth of the progeny, has been obtained by crossing breeds in some other kinds of livestock, it might reasonably be expected that crossing the dairy breeds would produce similar results. This paper presents an analysis of the rate of growth of first-generation crossbred dairy calves as compared with the growth standards for purebreds.

EXPERIMENTAL PROCEDURES

A cross-breeding project with dairy cattle was set up early in 1939 by the Bureau of Dairy Industry at the Agricultural Research Center, Beltsville, Md. The breeds involved are Holstein, Jersey, Guernsey, and Red Dane. Red Dane cattle rank next to Holsteins in size, mature cows weighing from 1,300 to 1,350 pounds. They are shorter legged, somewhat blockier, and have a tendency to be proportionately longer-bodied than the other dairy breeds.

The following crosses have been made: Holstein × Jersey, Jersey × Holstein, Holstein × Guernsey, Holstein × Red Dane, Red Dane × Holstein, Jersey × Red Dane, Red Dane × Jersey, and Red Dane × Guernsey. The first breed mentioned in each cross is that of the sire, the second that of the dam. Early in the experiment a few Jersey × Guernsey crosses were made, but the crossbred progeny are too small in number to be considered in this analysis. Only in the Holstein × Jersey combinations are the numbers large enough for separate studies of the reciprocals. Unless otherwise stated the data are for female calves only.

All crossbred calves in this study were raised by the same procedure as is used in raising calves in the breeding herds at Beltsville, which is as follows:

The feeding and management of the calves conform to good dairy practice. The calves are separated from the dams shortly after birth and are hand-fed in small pens. Whole milk is fed for the first 4 to 8 weeks, depending on the vigor of the calf, after which skim milk is substituted. Skim-milk feeding is discontinued after 6 months of age. Starting at about 3 weeks of age, the calves are fed alfalfa hay and a small amount of grain. The grain mixture consists of corn, oats, wheat bran, and linseed meal. The grain ration is gradually increased to a maximum of 3 pounds daily by the time skim-milk feeding is discontinued. After 12 months of age the heifers are housed in a stanchion barn and, in addition to hay and grain, they are fed a small ration of corn silage in the winter and are pastured during the summer months.

Every effort is made to insure the health of the calves. They are kept under close observation at all times, and any ailment is given prompt veterinary treatment.

Few of the male calves are retained in the experimental herds; hence only their birth weights are available for consideration here. Monthly weights for the heifer calves are taken from birth, but the weights after 18 months will not be considered here because the heifers are bred first at about 15 months of age and the effect of pregnancy becomes too much of a disturbing influence after 18 months.

The measures of growth used in this analysis are the live weight and a set of five skeletal measurements as follows: Height at withers, width of fore chest, depth of fore chest, width of hips, and the total length from the withers to the pin bones. Live weight is an average of the weight on 3 consecutive days, taken at intervals of 30 days, up to 12 months of age. Thereafter, all heifers are weighed on the first day of each month, this weight being used as the weight of the animal for the age unit nearest to the dates on which the weighings were made. The skeletal measurements are taken at 6, 12, and 18 months of age.

For a number of years, these same measures of growth have been applied to the calves in the purebred Holstein, Jersey, and Red Dane herds at Beltsville. From the resulting data, standards of weights and measurements have been calculated for use in making comparisons between the purebred calves and the crossbreds of these three breeds.

A study of growth data from the Beltsville herds and of similar data gathered at other institutions makes it seem unsound to compare such data from one herd against growth standards calculated from other herds where conditions of feeding and management are somewhat different. For this reason, the only standards of growth used in this analysis are those developed from Beltsville data.

Table 1 shows the standards of live weight for purebred Holstein, Jersey, and Red Dane calves; and table 2 shows the standards of skeletal growth for these three breeds.

TABLE 1.—*Standards of live weight for purebred dairy calves*

Age (months)	Holsteins			Jerseys			Red Danes		
	Animals	Average weight	Coefficient of variability	Animals	Average weight	Coefficient of variability	Animals	Average weight	Coefficient of variability
	Number	Pounds	Percent	Number	Pounds	Percent	Number	Pounds	Percent
Birth	229	95.8±0.79	12.53	254	55.2±0.46	13.28	49	80.8± 1.56	13.53
1	224	118.6± .93	11.72	240	71.7± .60	12.91	48	106.6± 2.16	14.02
2	223	159.4±1.42	13.31	235	100.3±1.16	17.67	49	144.8± 3.14	15.18
3	223	211.1±1.58	11.15	229	136.9±1.21	13.38	49	192.7± 3.71	13.47
4	222	268.2±1.95	10.84	230	178.6±1 49	12.65	49	244.7± 4.28	12.24
5	221	330.7±2.24	10.05	229	226.3±1.73	11.58	48	298.6± 5.12	11.87
6	218	390 9±2.67	10.08	229	275.1±1.94	10.65	48	350.3± 5.46	10.80
7	217	449.1±2.90	9.50	224	323.2±2.26	10.49	48	397.6± 5.73	9.98
8	216	502.7±3.39	9.90	221	365.2±2.47	10.06	48	437.4± 5 42	8.58
9	215	553.6±3.75	9.92	219	404.2±2.67	9.78	48	479.5± 5.67	8 20
10	211	605.0±3.88	9.31	217	441.0±2.93	9.80	48	520.9± 6.09	8.10
11	210	654.9±4.08	9.03	215	477.4±2.88	8.84	48	559.4± 6.63	8.21
12	213	706.2±4.51	9.31	217	513.4±3.31	9.49	47	608.2± 6.61	7.46
13	210	735.5±4.81	9.47	211	534.8±3.36	9.14	43	643.7± 7.75	7.89
14	210	770.5±4.77	8.98	214	556.5±3.57	9.39	43	679.9± 8.59	8.29
15	209	805.6±4.81	8.63	212	579.3±3.77	9.49	43	720.3± 9.02	8.22
16	206	841.5±5.03	8.58	209	606.5±4.04	9.62	43	753.3± 9.22	8.03
17	206	876.4±5.19	8.50	206	631.2±4.33	9.84	41	785.9±10.10	8.23
18	206	913.5±5.51	8.66	205	655.3±4.41	9.65	38	824.7±10.53	7.87

TABLE 2.—*Standards of skeletal growth for purebred dairy calves*

HOLSTEINS

Age, and item of measurement	Animals	Average measurement	Coefficient of variability	Age, and item of measurements	Animals	Average measurement	Coefficient of variability
	Number	Centimeters	Percent		Number	Centimeters	Percent
6 months:				12 months—Continued			
Height at withers	184	101.8±0.23	3.02	Width of hips	182	40.0±0.12	4.10
Width of fore chest	184	28.3±0.14	6.58	Total length	172	112.9±0.27	3.12
Depth of fore chest	184	46.9±0.14	4.12	18 months:			
Width of hips	184	30.5±0.12	5.10	Height at withers	173	126.4±0.22	2.30
Total length	172	92.3±0.29	4.11	Width of fore chest	173	40.7±0.19	6.19
12 months:				Depth of fore chest	173	64.5±0.16	3.22
Height at withers	182	117.9±0.21	2.40	Width of hips	173	45.7±0.13	3.72
Width of fore chest	182	36.4±0.17	6.20	Total length	163	124.0±0.30	3.04
Depth of fore chest	182	58.2±0.14	3.32				

JERSEYS

6 months:				12 months—Continued			
Height at withers	193	95.5±0.24	3.48	Width of hips	198	36.3±0.11	4.10
Width of fore chest	193	23.4±0.14	8.08	Total length	170	106.0±0.24	2.97
Depth of fore chest	193	44.4±0.13	4.09	18 months:			
Width of hips	193	27.4±0.11	5.55	Height at withers	191	117.2±0.20	2.31
Total length	164	87.4±0.09	1.35	Width of fore chest	191	34.4±0.17	6.74
12 months:				Depth of fore chest	191	60.8±0.13	3.03
Height at withers	198	110.0±0.20	2.57	Width of hips	191	41.2±0.13	4.28
Width of fore chest	198	30.8±0.16	7.17	Total length	165	115.6±0.27	2.99
Depth of fore chest	198	55.1±0.12	2.94				

RED DANES

6 months:				12 months—Continued			
Height at withers	58	97.5±0.38	2.95	Width of hips	60	38.4±0.19	3.87
Width of fore chest	58	27.6±0.21	5.91	Total length	60	107.3±0.38	2.74
Depth of fore chest	58	45.8±0.21	3.51	18 months:			
Width of hips	58	29.9±0.20	5.03	Height at withers	57	118.8±0.36	2.30
Total length	58	90.0±0.50	4.19	Width of fore chest	57	38.3±0.32	6.25
12 months:				Depth of fore chest	57	61.6±0.24	2.88
Height at withers	60	110.9±0.31	2.18	Width of hips	57	44.8±0.26	4.38
Width of fore chest	60	33.8±0.27	6.15	Total length	57	117.8±0.41	2.60
Depth of fore chest	60	55.5±0.21	2.88				

The groups of crossbreds are relatively small; therefore, a statistical test is necessary to determine the significance of any apparent evidence of heterosis. For this purpose Student's *t* test as described by Snedecor [2] is employed, using as a hypothesis, or expected value, the mean between the two parent breed averages for outcross females for each weight or measurement. This mean hereafter will be referred to as the "expected" weight or measurement. Thus, this test is simply a measure of the significance of the difference between the crossbred averages and the mean of the two parent breed averages.

GROWTH IN LIVE WEIGHT

The data on live weight are given in tables 3, 4, and 5, and represent three different two-breed combinations.

TABLE 3.—*Live weight data on Jersey × Holstein calves and Holstein × Jersey calves*

CALVES FROM HOLSTEIN DAMS

Age (months)	Animals	Average weight (A)	Coefficient of variability	Average weight of dams	Mean of parent breed averages (B)	Difference (A − B)
	Number	*Pounds*	*Percent*	*Pounds*	*Pounds*	*Pounds*
Birth	8	76.6±2.63	9.71	86.0±3.14	75.5	1.1
1	8	99.6±4.01	11.38	115.9±3.97	95.1	4.5
2	8	128.9±2.71	5.95	157.1±5.41	129.9	−1.0
3	8	175.9±2.67	4.30	203.0±6.65	174.0	1.9
4	8	225.5±4.77	5.99	254.7±8.15	223.4	2.1
5	8	276.9±7.53	7.70	311.0±8.59	278.5	−1.6
6	8	330.4±7.55	6.47	370.7±9.99	333.0	−2.6
7	8	387.1±9.88	7.22	428.0±8.68	386.1	1.0
8	8	432.1±9.67	6.47	484.1±9.94	434.0	−1.9
9	8	473.9±12.87	7.69	532.4±7.52	478.9	−5.0
10	8	511.6±14.80	8.19	577.9±12.85	523.0	−11.4
11	8	548.6±15.14	7.81	620.6±14.64	566.2	−17.6
12	8	594.8±13.61	6.48	660.0±22.67	609.8	−15.0
13	8	621.0±13.96	6.36	723.0±14.03	635.2	−14.2
14	8	648.0±13.12	5.73	754.6±14.85	663.5	−15.5
15	8	687.5±7.67	3.16	782.6±10.16	692.5	−5.0
16	8	711.5±8.26	3.28	816.0±10.13	724.0	−12.5
17	8	742.0±13.41	5.12	846.3±17.69	753.8	−11.8
18	8	785.5±12.60	4.54	858.7±18.18	784.4	1.1

CALVES FROM JERSEY DAMS

Age (months)	Animals	Average weight (A)	Coefficient of variability	Average weight of dams	Mean of parent breed averages (B)	Difference (A − B)
Birth	10	74.9±2.47	10.44	51.9±2.19	75.5	−0.6
1	10	93.5±2.57	8.67	68.0±2.30	95.1	−1.6
2	10	129.0±3.16	7.74	92.3±3.54	129.9	−.9
3	10	179.9±5.02	8.81	123.3±4.49	174.0	5.9
4	10	238.5±5.32	7.05	159.6±5.57	223.4	15.1*
5	10	299.0±5.36	5.67	200.3±7.54	278.5	20.5**
6	10	356.7±4.52	4.01	240.7±9.84	333.0	23.7**
7	10	416.1±5.51	4.19	281.3±12.36	386.1	30.0**
8	10	460.2±7.21	4.95	310.6±14.18	434.0	26.2**
9	10	496.0±7.55	4.81	337.6±17.41	478.9	17.1*
10	10	543.8±10.28	5.98	366.0±18.90	523.0	20.8
11	10	578.9±13.42	7.32	395.6±23.20	566.2	12.7
12	10	629.4±12.91	6.48	431.3±23.01	609.8	19.6
13	10	666.8±14.14	6.70	458.9±19.58	635.2	31.6
14	10	687.8±12.58	5.78	481.7±20.12	663.5	24.3
15	10	717.0±15.17	6.69	497.3±26.46	692.5	24.5
16	10	742.7±13.90	5.91	534.9±24.58	724.0	18.7
17	10	790.6±18.21	7.28	547.3±28.18	753.8	36.8
18	10	822.2±16.10	6.19	574.1±22.88	784.4	37.8*

*Significant difference. **Highly significant difference.

[2] SNEDECOR, G. W. STATISTICAL METHODS. Ed. 4, Rev. ed., 485 pp. illus. Ames, Iowa, 1946.

TABLE 4.—*Live weight data on Red Dane × Holstein calves and on Holstein× Red Dane calves*

Age (months)	Animals	Average weight (A)	Coeffi-cient of varia-bility	Mean of parent breed averages (B)	Differ-ence (A−B)
	Number	*Pounds*	*Percent*	*Pounds*	*Pounds*
Birth	13	94.0±3.06	11 77	88 3	5.7
1	12	113 3±3 07	9 39	112.6	.7
2	13	154.0±4.20	9.84	152.1	1.9
3	13	206.2±4 84	8.48	201 9	4 3
4	13	260.9±4 04	5.59	256.5	4.4
5	13	316.5±5 17	5 89	314.7	1.8
6	13	368.3±5 77	5 65	370 6	−2.3
7	13	413.8±6 43	5.61	423.4	−9.6
8	11	466.6±9.82	6.99	470 0	−3.4
9	11	520 6±10 58	6 75	516.5	4.1
10	10	571.9±13.22	7.31	563.0	8.9
11	10	620 7±11 75	5 98	607 2	13 5
12	10	678.6±11 38	5.30	657 2	21.4
13	10	714.3±12.01	5 31	689 6	24.7
14	10	744.0±14 44	6 13	725.2	18.8
15	10	772.8±9 80	4.01	763.0	9.8
16	10	802 4±10.51	4.14	797.4	5.0
17	9	833 6±16 81	6.05	831.1	2 5
18	8	877 0±14.36	4.63	869.1	7.9

TABLE 5.—*Live weight data on Red Dane × Jersey calves and on Jersey ×Red Dane calves*

Age (months)	Animals	Average weight (A)	Coeffi-cient of variabil-ity	Mean of parent breed averages (B)	Differ-ence (A−B)
	Number	*Pounds*	*Percent*	*Pounds*	*Pounds*
Birth	8	70.5±2.95	11.38	68.0	2.5
1	8	90.1±3.71	11.66	89.1	1 0
2	8	128.0±4.74	10.48	122.6	5 4
3	8	174.9±6.48	10.49	164.8	10.1
4	8	223.4±7.89	9.89	211.7	11.7
5	8	276 8±8.30	8.49	262.5	14.3
6	8	329.6±8.53	7.32	312.7	16.9
7	8	380.8±10.54	7.83	360 4	20.4
8	8	428 4±13.81	9.13	401.3	27.1
9	8	468.6±13.88	8.38	441.9	26 7
10	8	511.1±15 41	8.53	480.9	30.2
11	8	546.9±20.04	10 37	518.4	28 5
12	8	590.9±19.89	9.53	560.8	30.1
13	8	618.5±22.34	10.22	589.3	29.2
14	8	650 9±20.29	8.82	618.2	32.7
15	8	677.0±21.98	9.19	649.8	27.2
16	8	707 3±20.65	8.26	679.9	27.4
17	7	739.9±28.98	10.38	708 5	31.4
18	7	765.7±28.62	9.90	740.0	25.7

The first point of interest in these tables is the birth weight of the crossbred calves. Apparently there is not enough difference in size between the parent breeds used here to exhibit the phenomenon described by Hammond,[3] where the size of the dam controls the size of the offspring. In the combinations representing the Holstein and Jersey breeds, in which there is the greatest contrast in size of the parent breeds, the female calves from Holstein sires and Jersey dams

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[3] HAMMOND, J. PHYSIOLOGICAL FACTORS AFFECTING BIRTH WEIGHT. Nutr. Soc. Proc. 2: 8–12, illus. 1944.

had an average birth weight of 74.9 pounds and those from Jersey sires and Holstein dams averaged 76.6 pounds, whereas the mean of the birth weight averages for the parent breeds was 75.5 pounds (table 3).

There was somewhat more difference in birth weight between the Jersey×Holstein males and the Holstein×Jersey males than between females of the same crosses. Seven males from Holstein sires and Jersey dams averaged 76.3±2.37 pounds at birth, and six males from Jersey sires and Holstein dams averaged 82.0±3.87 pounds. This difference is not statistically significant.

It is interesting to note further that the Holstein×Jersey calves from Jersey dams developed more rapidly after 3 months of age than did the reciprocal cross from Holstein dams (table 3).

In general it can be said that the cross breds have a tendency to be slightly heavier than the expected weight. This difference is not statistically significant in a majority of cases.

Reports of previous cross-breeding work[4][5] indicate there is more evidence of heterosis when two large breeds are crossed than when a large breed and a small one are crossed. The work at Beltsville has not borne out this theory. The crossbreds from the two largest breeds, the Holstein and the Red Dane, show the least average increase over the expected weight of any of the crosses. At 6, 7, and 8 months of age, the Holstein×Red Dane crossbreds averaged slightly less than the expected weight.

The figures show some interesting breed interactions in live weight. In the Holstein×Jersey crossbreds (table 3) the period of most rapid growth is from 4 to 8 months. In the Red Dane×Jersey crossbreds (table 5) growth seems to be quite uniform from the second month on. The Guernsey crosses afford the most striking examples of different breed interactions; however, these are treated separately in another section.

The coefficients of variability indicate that there is somewhat less variance in the crossbred groups than in the large purebred groups from which the weight standards were calculated.

SKELETAL GROWTH

The data on skeletal growth are given in tables 6, 7, and 8. Crosses involving Guernseys are omitted from this phase of the study because there was no suitable set of standards for purebred Guernseys.

[4] HAMMOND, J. ON THE RELATIVE GROWTH AND DEVELOPMENT OF VARIOUS BREEDS AND CROSSES OF CATTLE. Jour. Agr. Sci. [England] 10: [233]–289. 1920.

[5] COLE, L. J. THE WISCONSIN EXPERIMENT IN CROSSBREEDING CATTLE. World's Dairy Cong. Proc., Washington, Philadelphia, and Syracuse, 1923, 2: 1383–1388. 1924.

TABLE 6.—*Skeletal growth data on Jersey × Holstein calves and on Holstein × Jersey calves*

CALVES FROM HOLSTEIN DAMS

Age, and item of measurement	Animals	Average measurement (A)	Coefficient of variability	Mean of parent breed averages (B)	Difference (A−B)
	Number	Centimeters	Percent	Centimeters	Centimeters
6 months:					
Height at withers	8	99.0±.65	1 86	98.6	0.4
Width of fore chest	8	26 4±.29	3.10	25.8	.6
Depth of fore chest	8	45.8±.45	2 80	45.7	.1
Width of hips	8	29.6±.33	3.19	28.9	.7
Total length	8	91.6±.88	2.71	89.9	1.7
12 months:					
Height at withers	8	112.8±.86	2.17	114.0	−1.2
Width of fore chest	8	32.4±.66	5.76	33.6	−1.2
Depth of fore chest	8	55 7±.62	3.13	56.7	−1.0
Width of hips	8	38.8±.61	4 47	38.2	.6
Total length	8	110 2±.80	2.05	109.4	.8
18 months:					
Height at withers	8	120.6±.83	1 94	121.8	−1.2
Width of fore chest	8	36.2±.70	5.50	37.5	−1.3
Depth of fore chest	8	61.5±.52	2.41	62.7	−1.2
Width of hips	8	44.2±.57	3.63	43.5	.7
Total length	8	120.7±1.20	2.80	119.8	.9

CALVES FROM JERSEY DAMS

Age, and item of measurement	Animals	Average measurement (A)	Coefficient of variability	Mean of parent breed averages (B)	Difference (A−B)
6 months:					
Height at withers	10	100.4±.70	2 20	98.6	1.8*
Width of fore chest	10	26.3±.39	4.66	25.8	.5
Depth of fore chest	10	46.8±.30	2.02	45.7	1.1**
Width of hips	10	29.8±.29	3.03	28.9	.9*
Total length	10	93.4±.92	3.11	89.9	3.5**
12 months:					
Height at withers	10	115.7±.94	2.57	114.0	1.7
Width of fore chest	10	33.3±.32	3 05	33.6	−.3
Depth of fore chest	10	57.4±.42	2.32	56.7	.7
Width of hips	10	39.4±.28	2.26	38.2	1.2**
Total length	10	112.9±1.18	3.31	109.4	3.5*
18 months:					
Height at withers	10	123.5±1.09	2.79	121.8	1 7
Width of fore chest	10	38.0±.35	2.92	37.5	.5
Depth of fore chest	10	63.4±.49	2 43	62.7	.7
Width of hips	10	44.9±.48	3.36	43.5	1.4*
Total length	10	122 2±1.35	3.48	119.8	2.4

*Significant difference.   **Highly significant difference.

TABLE 7.—*Skeletal growth data on Holstein × Red Dane calves and on Red Dane × Holstein calves*

Age, and item of measurement	Animals	Average measurement (A)	Coefficient of variability	Mean of parent breed averages (B)	Difference (A−B)
	Number	*Centimeters*	*Percent*	*Centimeters*	*Centimeters*
6 months:					
Height at withers	13	100.8±.62	2.23	99.6	1.2
Width of fore chest	13	27.8±.39	5.01	27.9	−0.1
Depth of fore chest	13	46.8±.32	2.47	46.4	.4
Width of hips	13	30.2±.37	4.46	30.2	0
Total length	13	92.7±.72	2.82	91.2	1.5
12 months					
Height at withers	10	115.1±.61	1.68	114.4	.7
Width of fore chest	10	35.1±.63	5.70	35.1	0
Depth of fore chest	10	57.2±.25	1.37	56.8	.4
Width of hips	10	39.8±.37	2.97	39.2	.6
Total length	10	110.8±.80	2.28	110.1	.7
18 months:					
Height at withers	10	122.8±.74	1.90	122.6	.2
Width of fore chest	10	39.7±.26	2.04	39.5	.2
Depth of fore chest	10	62.7±.49	2.46	63.0	−.3
Width of hips	10	45.6±.55	3.79	45.2	.4
Total length	10	121.8±.79	2.04	120.9	.9

TABLE 8.—*Skeletal growth data on calves by Red Dane sires and out of Jersey dams*

Age, and item of measurement	Animals	Average measurement (A)	Coefficient of variability	Mean of parent breed averages (B)	Difference (A−B)
	Number	*Centimeters*	*Percent*	*Centimeters*	*Centimeters*
6 months:					
Height at withers	7	96.8±0.69	1.88	96.5	0.3
Width of fore chest	7	26.9±.61	6.02	25.5	1.4
Depth of fore chest	7	45.6±.42	2.46	45.1	.5
Width of hips	7	28.9±.63	5.79	28.7	.2
Total length	7	89.8±.69	2.03	88.7	1.1
12 months:					
Height at withers	8	110.6±1.19	3.04	110.5	.1
Width of fore chest	8	34.4±.77	6.48	32.3	1.1
Depth of fore chest	8	55.7±.47	2.40	55.2	.5
Width of hips	8	38.3±.62	4.57	37.3	1.0
Total length	8	107.8±1.11	2.92	106.6	1.2
18 months:					
Height at withers	7	118.4±1.46	3.27	118.0	.4
Width of fore chest	7	38.1±.70	4.85	36.3	1.8[*]
Depth of fore chest	7	61.6±.63	2.72	61.2	.4
Width of hips	7	44.3±.74	4.41	43.0	1.3
Total length	7	117.8±1.68	3.78	116.7	1.1

[*]Significant difference.

The same general trends are found in the skeletal measurements as in the live weight data. The most noticeable feature is that the Holstein × Jersey and Jersey × Holstein crossbreds seem to be somewhat rangier than the crossbreds of the other breeds, particularly at 6 months of age. The Holstein × Red Dane crossbreds show the least heterosis of any of the crossbreds. Again, as in live weight, the Red Dane × Jersey crossbreds are quite uniform in skeletal growth.

Figures 1 to 5 show the general conformation of the crossbreds resulting from various crosses. These photographs, all taken at 6 months of age, are of calves chosen as the most representative of their respective groups with regard to size and general growth habits.
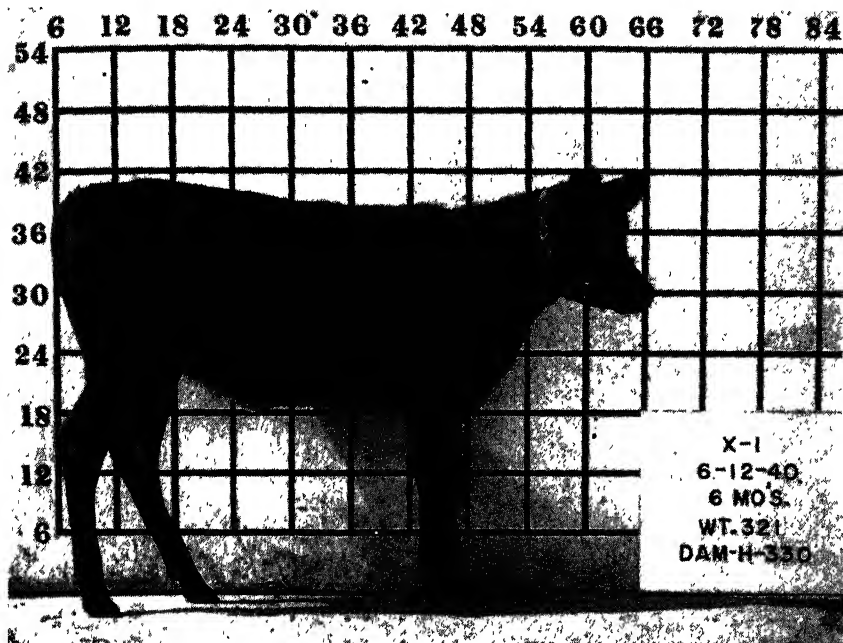
FIGURE 1.—Crossbred calf (Herd No. X-1) by a Jersey sire and out of a Holstein dam.
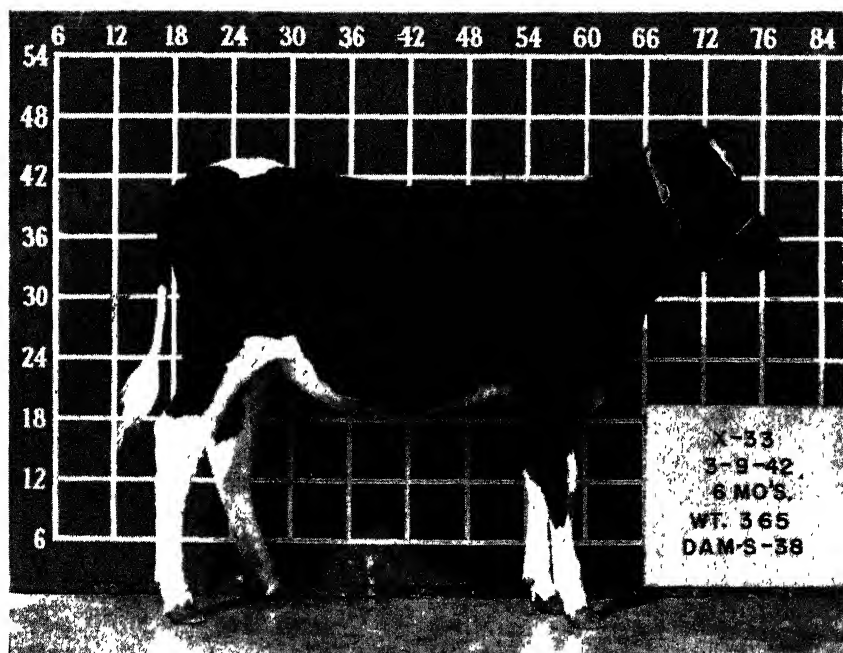
FIGURE 2.—Crossbred calf (Herd No. X-33) by a Holstein sire and out of a Guernsey dam.
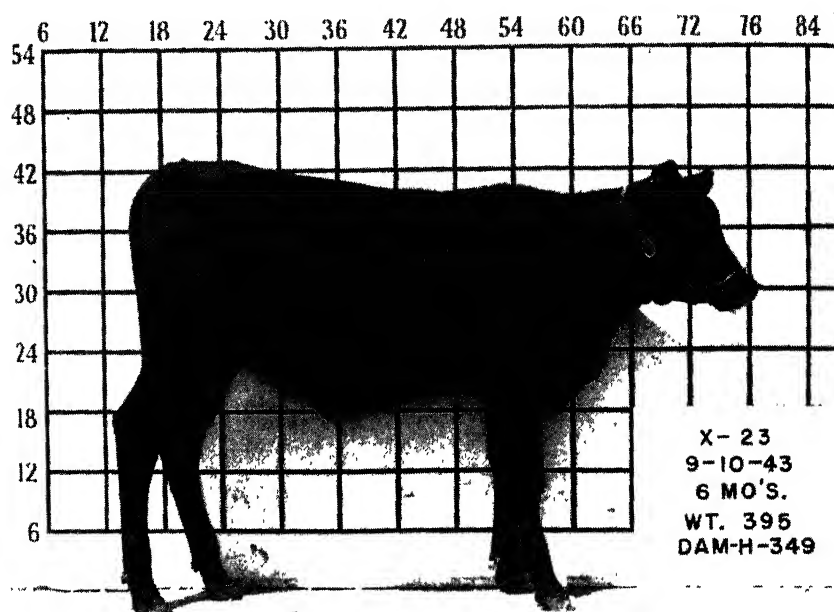
FIGURE 3.—Crossbred calf (Herd No. X-23) by a Holstein sire and out of a Red Dane dam.
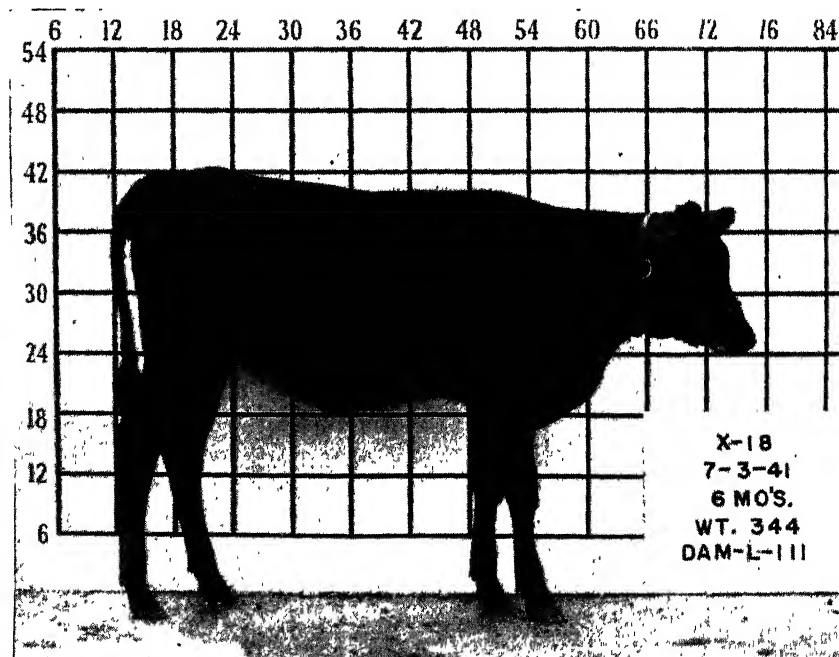
FIGURE 4.—Crossbred calf (Herd No. X-18) by a Red Dane sire and out of a Jersey dam.
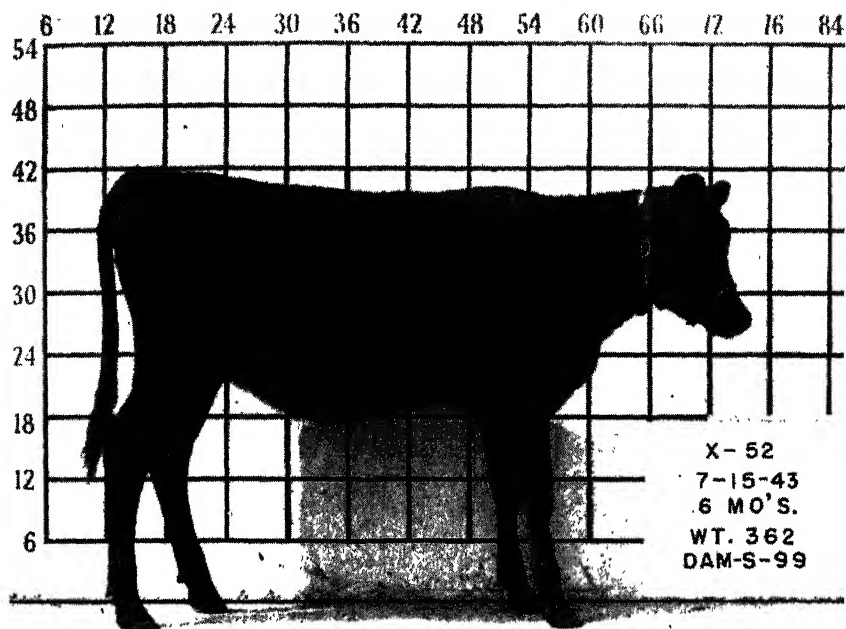
FIGURE 5.—Crossbred calf (Herd No. X-52) by a Red Dane sire and out of a Guernsey dam.

GROWTH OF GUERNSEY CROSSES

The growth in live weight of the crosses involving Guernseys (Holstein × Guernsey and Red Dane × Guernsey) appears to follow a somewhat different pattern from that of the other crosses discussed thus far. For this reason an analysis of the data for these crosses seems desirable, even though no suitable standard for purebred Guernseys is available for comparison.

A study of the data indicated that the slope of the growth curve for the Holstein × Guernsey crossbreds differed from that of the Red Dane × Guernsey crossbreds; nor did these curves slope as might be expected judging from the data on the purebred Holsteins and Red Danes. To illustrate this, a calculation was made showing the percentage of the final weight gained during each month from birth to 18 months of age. As used here the "final weight" is the mean of the weights at 17, 18, and 19 months of age, which reduces the chance of error that could be introduced by using a single 18-month average weight. These calculations were made for the Holstein × Guernsey and Red Dane × Guernsey crossbreds and purebred Holstein and Red Dane groups. The figures are summarized in table 9.

TABLE 9.—*Comparative rate of growth of Holstein ✕ Guernsey calves and Red Dane ✕ Guernsey calves*

Period of growth (average)	Proportion of total growth per month			
	Holstein	Red Dane	Holstein ✕ Guernsey	Red Dane ✕ Guernsey
	Percent	*Percent*	*Percent*	*Percent*
Birth to 12 months	5 62	5 42	5 34	5. 52
12 to 18 months	3. 70	4. 22	4 35	3. 68

It will be noted that in the purebred groups the rate of growth of the Holsteins was relatively higher than that of the Red Danes for the first 12 months, whereas from 12 to 18 months the Red Danes gained at a relatively faster rate than did the Holsteins. When each of these two breeds was crossed with Guernseys the trend was exactly the opposite, the higher early rate of growth being attained by the Red Dane ✕ Guernsey crossbreds while the Holstein ✕ Guernsey crossbreds grow relatively faster during the later period.

The total growth for the two periods also affords an interesting comparison. The Holsteins gained 67.47 percent of their final weight from birth to 12 months, and 22.20 percent from 12 to 18 months. The corresponding figures for the other groups are: Red Danes, 64.99 and 25.31 percent; Holstein ✕ Guernsey, 64.12 and 25.90 percent; Red Dane ✕ Guernsey, 66.21 and 22.05 percent. It will be noted that the percentages for the Red Dane ✕ Guernsey crossbreds closely approach those of the purebred Holsteins, while the Holstein ✕ Guernsey figures more nearly approximate those of the purebred Red Danes.

Still another method may be used to illustrate these different breed interactions. For this method, the assumption is made from the data in tables 3, 4, and 5 that the weights of the crossbreds will fall approximately midway between the parent breed averages. On the basis of this assumption, the Guernsey average weights can be roughly estimated from the weights of the crossbreds and the average weights of the other parent breed. Table 10 shows the estimates of the Guernsey average weights made from the data on the crosses with Holsteins and with Red Danes.

TABLE 10.—*Estimate of Guernsey weight averages*

Age (months)	Estimate of Guernsey weight averages	
	From Holstein ✕ Guernsey crossbreds	From Red Dane ✕ Guernsey crossbreds
	Pounds	*Pounds*
Birth	66	91
6	260	330
12	512	616
18	739	734

The wide divergence of these estimates makes it obvious that the assumption made above is not valid in the case of these crosses. The

relatively rapid rate of early growth in the Red Dane×Guernsey crossbreds is again apparent in this tabulation.

We have no explanation for the different breed interactions described here. Although the figures presented in tables 9 and 10 do not lend themselves to statistical analysis, the data have been explored thoroughly, and it seems very unlikely that the differences are due entirely to chance variation in the small groups of crossbreds. Probably there will be no satisfactory explanation for these breed interactions until much more is known about cattle genetics.

The data in this section indicate that the growth obtained by crossing two given breeds does not necessarily afford an accurate prediction of the result of crossing either breed with other breeds.

SUMMARY

Growth studies are presented on first-generation crossbred dairy calves, representing crosses of Holstein, Jersey, Guernsey, and Red Dane cattle. The measures of growth used are live weight and a set of five skeletal measurements.

There is some indication that the crossbreds tend to be slightly larger than the mean of the parent breed averages. Heterosis is indicated in some groups by the fact that the crossbreds are significantly larger than the expected weight or measurement, but this does not hold true for all crossbred calves.

Some interesting differences in breed interactions are shown, particularly in the Guernsey crosses.

THE EFFECTS OF AIRPLANE DDT APPLICATIONS ON CITRUS GROVES IN FLORIDA [1] [2]

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INTRODUCTION

During the war years the Army and Navy made extensive use of airplanes for the dispersal of DDT for mosquito and fly control. Linquist *et al.* (*1*) [4] stated that a 5-percent solution of DDT applied at 2–3 quarts per acre would give satisfactory adult mosquito control. As the result of both experimental and practical experience, counties and cities have begun to engage in this type of pest-control program. DDT has shown itself to be most beneficial when used in this manner. However, instances have been reported where DDT apparently was having some detrimental effects.

Griffiths and Thompson (*2*) and Griffiths and Stearns (*3*) reported a reduction in the parasites and predators of Florida red scale (*Chrysomphalus aonidum* (L.)) and a subsequent abnormal population increase for this pest where DDT was applied with conventional spray equipment on citrus trees in Florida. Woglum (*4*) reported complications in citricola scale (*Coccus pseudomagnoliarum* (Kuw.)) and cottony-cushion scale (*Icerya purchasi* Mask) control in California citrus groves. More recently Morrill and Otanes (*5*) have reported scale and mealybug infestations in and around Manila which they believed were attributable to the regular application of DDT over that city. Consequently, the widespread use of airplane dispersed DDT in certain coastal areas of Florida was viewed with apprehension by some citrus growers.

EXPERIMENTAL PROCEDURE

The following is an account of observations made in citrus groves in and around Cocoa in Brevard County on the east cost of Florida. Here, the county had undertaken an extensive campaign for mosquito control. This area was typical of others in Florida where DDT was

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sprayed from airplanes, and it is believed that the observations made here would be applicable to other citrus growing areas of the State.

In the Brevard County operations, flights were scheduled according to "bite counts" so that some areas were more frequently sprayed than others. The material used consisted of 35 pounds of technical DDT per 100 gallons of spray and this was dispersed at about 10 gallons per minute by a plane that averaged 70 miles per hour. As nearly as possible an attempt was made to spread the material over approximately a 100-foot swath. With these figures as a basis, it may be calculated that about one quarter of a pound of DDT was distributed over an acre area. If it is assumed that this was an absolutely flat surface, then less than 2.8 μg of DDT was deposited per cm^2. However, since an acre of vegetative growth comprises many acres of surface, only an infinitesimally small amount of actual DDT would be expected to be deposited on any surface at any application.

Determinations of the actual amounts of DDT present following the airplane sprayings were made according to Redd's modification of a method by Schecter et al. (*6*). This method determines the total amount of DDT present both inside and on the surface of the leaf. The procedure included the following technique: Twenty-five leaves were selected at random and a round disk was punched from each leaf. Each disk represented an area of 1 square inch (i. e., a total leaf surface, upper and lower, of 2 square inches). These disks were extracted for 1 hour with 150 ml. of petroleum either (boiling point 35° to 60° C.) In a Soxhlet extractor. The volume was reduced by evaporation to 15 ml. and the material transferred to a 50-ml. centrifuge tube.

The extraction flask was washed twice with small portions of the ether and the washings were added to the centrifuge tube. The ether was evaporated, and the tubes cooled in water. Five ml. of nitrating mixture composed of 1 to 1 volume of fuming nitric acid (specific gravity 1.50) and concentrated sulfuric acid (specific gravity 1.84) were added, and the tubes were heated in a water bath for 1 hour at 100° C. After cooling the tubes in cold water, 25 ml. of ice water were added. The solution was transferred to a 60-ml. separatory funnel, and after the tubes had been thoroughly washed, the wash was added to the funnel. This mixture was then extracted with 20 ml. of a solution containing 60-percent petroleum ether and 40-percent benzene. It was shaken vigorously for 1 minute and after separation of the layers, the aqueous portion was discarded. The solution was washed with 5 ml. of water and the aqueous portion discarded. After a second washing with 10 ml. of 2-percent sodium hydroxide the aqueous portion was again discarded.

Following this a third washing with water was performed. The petroleum etherbenzene portion was then transferred to a dry flask, placed in a water bath and evaporated to dryness under vacuum. Five ml. of anhydrous benzene and 10 ml. of sodium methylate solution (10 gr. ± 0.1 gr. per 100 ml. of anhydrous methanol) were added to the flask and thoroughly mixed. The blue color was read within 15 minutes and the amount of p,p-DDT was calculated from standard-curves prepared from pure tetranitro p,p-DDT using 590-mμ filter.

Ebling (7) reported that DDT in kerosene emulsions was absorbed into the citrus leaf. In order to check this report under Florida conditions a procedure was worked out to determine whether DDT was absorbed by the leaves. It was found that when DDT and oil emulsions were sprayed on citrus trees some DDT was absorbed by the leaf. The absorbed DDT was determined in the following manner: Twenty-five leaves selected at random were placed in a 1-percent solution of Nytron, a highly active surface agent, and thoroughly scrubbed with a soft brush. Following this treatment they were rinsed several times with tap water and with distilled water and allowed to air dry. The leaves were then analyzed for DDT by the procedure described above. The DDT found at this analysis was considered to have been absorbed into the inside of the leaf. Any difference between this amount and the total amount found on a similar sample which had not been washed was considered to represent the DDT on the surface of the leaf.

RESULTS

Table 1 presents data on p,p-DDT deposits on citrus foliage at various locations and various dates during the 1947 mosquito spraying season. In some cases the DDT is expressed both as internal and total DDT. The part of the tree from which the samples were taken is indicated as well as the number of times sprayed by county planes and the date of the last spraying.

Several facts are evident from table 1. All DDT deposits were within a range where experimental error was as great as the differences noted. Thus, any figure under 0.5 μg DDT per cm^2 is not a reliable one. However, certain trends are evident. On August 18 and 19 samples were taken before and after an application. The trees from which these samples were taken were located inside the city limits of Cocoa and in this area heavier than average applications occurred. Apparently about 0.10–0.15 μg DDT was deposited per cm^2. Reference to the entire set of figures indicates that although this amount might be applied at one application, in no instance did the total amount on the leaf accumulate to as much as 0.3 μg per cm^2. Apparently the dissipation between sprayings was as great or greater than the amount deposited. The data of November 22 show marked irregularity, but this is easily explained by the low levels of DDT being considered. However, these data appear to indicate that most of the DDT at that date was internal. In any case, in spite of as many as 10 applications, DDT was still present in only minute amounts. Griffiths and Stearns stated that damage to the predaceous and parasitic insects that helped control Florida red scale was accomplished when DDT deposits were higher than 0.5 μg cm^2. Thus, in no instance did deposits attain a level which would be expected to adversely affect such beneficial populations.

TABLE 1.—*DDT deposits on citrus foliage following airplane applications, 1947*

Date	Grove	Location on tree	µg of DDT per cm² Internal	Total	Number of times sprayed by county planes	Date of last spray
May 26	A-1	Top		0	0	
	A-2	do		0	0	
	A-3	do		0	0	
	B	Side		0	0	
	C	do		0	0	
July 17	A-1	Top		0 13	4	July 12
Aug. 12	do	do		.12	6	Aug. 8
	do	Side		.11	6	Do.
	A-2	Top		.06	6	Do.
	do	Side		.06	6	Do.
	A-3	Top		.06	6	Do.
	do	Side		.06	6	Do.
Aug. 18	D	Top		.17	4	July 16
	do	Side		.12	4	Do.
Aug. 19	D	Top		.28	5	Aug. 19
	do	Side		.28	5	Do.
Nov. 22	A-1	Top	0.09	.12	10	Oct. 21
	A-3	do	.04	.03	10	Do.
	B	Side		.12	10	Oct. 30
	C	do		.03	10	Do.
	D	Top	.18	.10	7	Oct. 9
	E	do	.09	.03	7	Do.
	F	do	.12	.06	7	Do.

Notes on parasitic and predaceous populations were made during the summer and fall in groves sprayed with DDT. Grove A is of particular interest. This grove was located on Merritt Island adjacent to Indian River. The owner lived on the grove and the trees about his house received considerably more DDT than did those at the far side of the grove. Samples labeled A-1 in table 1 were taken adjacent to the house. A-3 was at the far side of the grove and A-2 was midway between A-1 and A-3. In August, counts of Florida red scale parasites were made both at A-1 and A-3 locations. No parasites were recorded. At the same time more twice-stabbed lady beetles (*Chilocoris stigma* (Say)) appeared to be present at A-3 than at A-1 location and there was definitely more evidence of feeding holes in red scale armors at A-3 than A-1. Florida red scale was common, but not abundant, at both locations. The fact that no parasites were found in the sample examined is probably of no significance, since other samples taken from the east coast of Florida during July and August failed to show any parasites either. Red scale populations were generally low at that time and apparently parasite density was correspondingly low. In September, samples were again taken and this time one second stage parasite (presumably *Prospaltella aurantii* (How.)) was found.

A final survey was made on November 22 in groves A, D, and F. Evidences of parasitism were recorded as shown in table 2. No third stage parasites were found, but this was again in line with other groves sampled in other places on the east coast of the State. Second-stage parasitism was common and well within the range of normal expectancy for that time of the year. Not only were the parasites common, but twice-stabbed lady beetles were present and often numerous at all locations noted in table 1 for the November date of sampling.

TABLE 2.—*Parasitism of Florida red scale in November after 10 applications of DDT*

Grove	Location on tree	Second stage parasites		Third stage parasites	µg of DDT for cm ^{2}
		Live parasites,/100 live scale ^{a}	Evidences of parasites/^{b} 100 live scale		
D	Top	4	10	0	0.10
A-1	do	12	24	0	.12
A-1	Side	14	16	0	.12
A-3	Top	24	32	0	.04
F	do	20	20	0	.06

^{a} Live scale included only late second- and very early third-stage females.
^{b} Evidences of parasites included live larvae or pupae, dead ones, and emergence holes.

DISCUSSION

In considering the possible deleterious effects of airplane DDT spraying on Florida red scale control, several factors should be kept in mind. Apparently only small amounts of DDT were deposited on citrus foliage at any one application. These amounts were largely dissipated between applications so that there was no accumulation of DDT on the leaves. At the end of the spray season, there was no more DDT than early in the year, and in the fall most of the insecticide appeared to be on the inside of the leaf. At no time during the season was more than 0.3 µg per cm^2 recorded. This amount would not be considered as harmful to the insects which are parasitic or predaceous upon Florida red scale.

Observations on parasites and predators substantiated this conclusion since normal concentrations of lady beetles and parasites of second stage scale were found at the end of the season in November. The fact that no parasites of third stage scale were recorded may be discounted since the incidence of this parasite was low elsewhere along the east coast of Florida.

The only possibilities for detrimental effects from DDT would appear to be where an overzealous pilot regularly applied too much DDT to some area. Even then it would probably be difficult to get a deposit of DDT sufficiently high to adversely affect the insects that feed in or upon Florida red scale. This is evidenced by the fact that locations A-1, D, E, and F in table I were regularly sprayed with more than the specified amount of DDT. These facts would appear to justify the conclusion that DDT airplane spraying for mosquito control in Brevard County, Fla., was not detrimental to insect parasites and predators of Florida red scale in citrus groves and that from this standpoint, groves could be treated without fear of complications in red scale control.

SUMMARY AND CONCLUSIONS

In 1947 in Brevard County, Fla., airplanes were used extensively to distribute DDT for mosquito control. A study of the amount of p,p'DDT deposited on citrus foliage and of the effects of this DDT on the parasites and predators of Florida red scale in citrus groves

was made. No deposits of more than 0.3 μg of DDT per cm² were found and in November, parasitic and predaceous populations were apparently normal. Most of the DDT retained by the foliage appeared to have been absorbed into the leaf tissue. It is concluded that the spraying of DDT as practiced in Brevard County would have no detrimental effects on Florida red scale control in citrus groves.

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PALATABILITY TESTS ON POTATOES GROWN IN SOIL TREATED WITH THE INSECTICIDES BENZENE HEXACHLORIDE, CHLORDANE, AND CHLORINATED CAMPHENE [1]

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INTRODUCTION

The effectiveness of soil treatment with benzene hexachloride for the control of wireworm has been demonstrated at the Connecticut Agricultural Experiment Station.[2] Potato crops in Connecticut and other States are subject to considerable damage from wireworm, and the use of an effective insecticide would mean a substantial increase in the annual marketable yield.

Potatoes grown in soil treated with benzene hexachloride have an off-flavor according to numerous local consumers. The School of Home Economics at the University of Connecticut was asked to conduct palatability tests to determine whether the comments on off-flavor were justified. The first test, in the spring of 1947, showed that, at certain dosages, benzene hexachloride imparted a foreign flavor in the cooked potatoes.[3] The palatability tests were also carried out on the 1947 crop. The results of these tests are reported in this paper. In addition, these latter tests included potatoes grown in soil treated with chlorinated camphene, and in soil treated with chlordane. These chemicals were both being studied as insecticides. The purpose of these palatability tests was to determine whether the various insecticide treatments used for wireworm control affected the flavor of cooked potatoes.

PROCEDURE

Treated and untreated samples of potatoes, from both experimental and commercial fields, were supplied by the Connecticut Station. The tubers were stored in a dark basement room until used. The palatability panel for the taste tests consisted of staff members and students at the University of Connecticut.

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[2] Greenwood, Douglas E. benzene hexachloride and wireworm control. Jour. Econ. Ent. 40: 724–727. 1947.

[3] Black, M. R. the effect of using various treatments of the hexachlorocyclohexane insecticide on the palatability of cooked potatoes. Unpublished Paper, School of Home Economics, University of Connecticut. 1947.

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A uniform method of cooking was used in the nine tests. Two hundred gm. of peeled potatoes were cut into ⅛-inch slices and cooked in 300 gm. of boiling water until tender. The degree of doneness was determined with a cake tester and the cooking time averaged 15 to 20 minutes for all samples. Although parts of four or five potatoes were used for each sample, some of the inconsistencies observed may have been due to too limited sampling. After draining, they were placed on marked plates, mashed with a fork, and covered.

The tasting for all but two of the tests (Nos. 4 and 5) was done in this manner. The samples for each day were lettered and arranged in a random order. The tasters indicated the presence or absence of any off-flavor by plus (+) and minus (−) signs, respectively, and its intensity by (+), (++), etc. They also were asked to describe the flavor. Reference samples, identified as treated and untreated, were available to indicate to the tasters the nature of the off-flavor. In test No. 4 the samples were placed in order of the increasing dosage of the insecticide. The tasters were asked simply to check the presence or absence of any off-flavor. In test No. 5 the tasters were given eight samples (four treated and four untreated), arranged in random order, and were asked to group them into two groups of four identical samples. The potato samples tested are listed and described in table 1.

TABLE 1.—*Description of Potatoes Used in Palatability Tests*

Test No.	Variety	Insecticide	Dosage (pounds per acre)		Method of application
			Test samples	Reference samples	
1	Katahdin	Benzene hexachloride	0, 1, 2, 2½,[1] 8	0, 8	Spray.
2	do	do	0, 1, 2, 2½,[1] 8	0, 8	Do.
3	Green Mountain	Benzene hexachloride[2]	0, 7½, 50, 50	0	Dust.
4	do	do[2]	0, 7½, 50, 50		Do.
5	do	do[2]	0, 0, 0, 0, 50, 50, 50, 50		Do.
6	do	Pure gamma benzene hexachloride.	0, 0.25, 0.25, 0.25, 0.25	0, 25	Do.
7	Katahdin	Chlordane	0, 1, 2, 4, 8	0, 8	Spray.
8	do	Chlorinated camphene	0, 1, 2, 4, 8	0, 2	Do.
9	Green Mountain	do	0, 1, 1, 2, 2	0, 8	Do.

[1] Samples from commercial field.
[2] Soil treated in 1946; no treatment in 1947.

The method used to analyze the data depended upon the type of test. In the tests in which the subjects were asked to indicate the intensity of the off-flavor, the original markings were converted to numerical scores for analysis. These scores were those given by Fisher and Yates [4] for ranked data in series of 4 (−1.03, −0.30, 0.30, 1.03 in increasing order of off-flavor) or 5 (−1.16, −0.50, 0, 0.50, 1.16). Where two or more samples were marked alike, the corresponding scores were averaged. The scores were totaled for each sample and the statistical significance of the results in each test was assessed by an analysis of variance.

When only the presence or absence of an off-flavor was reported in a fixed order of tasting (test No. 4), the judgments on two successive

[4] FISHER, R. A., and YATES, F. STATISTICAL TABLES FOR BIOLOGICAL, AGRICULTURAL AND MEDICAL RESEARCH. Oliver and Boyd, Edinburgh. 1948.

samples were tested for independence by computing chi-square from
2×2 contingency tables, using Yates' correction for continuity. The
proportion of off-flavor judgments in the totals for treated and un-
treated samples were then compared in a similar fashion. In test No.
5 in which eight samples were divided into two groups of four each,
a frequency distribution was prepared of the observed number of
correct judgments and compared by chi-square with the number to be
expected if the subjects had been unable to distinguish between the
treated and untreated potatoes.

An effort was made to classify the tasters on the basis of their con-
sistency in duplicate tests Nos. 1 and 2 but the scores for any given
taster differed more from day to day than those of different tasters
on the same day. Since the tasters could not be classified as good or
poor, all scores were used in determining the totals in the benzene
hexachloride tests. All tasters' scores were included also in the totals
for chlordane and chlorinated camphene.

RESULTS AND DISCUSSION

BENZENE HEXACHLORIDE

The scores for tests Nos. 1 and 2 (table 2) indicate that the greater
the dosage of benzene hexachloride the stronger the off-flavor in
the potatoes. The mean scores differ significantly from one another
($P < 0.001$) and show an increase in value (except at the 1- and 2-pound
levels in test 1) as the dosage of the insecticide is increased.

The tasters who were able to detect the insecticide found the flavor
quite objectionable. They described it as strong, earthy, metallic,
bitter, medicinal, musty, and stinging or biting to the tongue. Some
found that the flavor persisted as long as 2 or 3 hours.

TABLE 2.—*Total scores showing extent of off-flavor in potatoes grown in soil
treated with benzene hexachloride at different dosages in pounds per acre*

Test No.	Number of tasters	Total scores at pounds-per-acre dosages of—				
		0	1	2	2½ [1]	8
1	16	−8.79	1.38	−5.65	5.69	7.36
2	27	−13.80	−6.96	5 34	6.71	8.75

[1] Sample from commercial field.

When benzene hexachloride was applied to the soil in the year
prior to that in which the potatoes were grown, the results were less
conclusive. In two tests Nos. 3 and 4 (tables 3 and 4), the tasters
were able to distinguish between samples that had been grown on
treated and untreated soil. In test No. 3 in which the random order
of tasting was followed, the scores differ significantly ($P < 0.001$)
from one another and are related to the dosage of the insecticide.
The difference between the two scores at the 50-pound level probably
indicates irregularity in the effect of the chemical on the individual
potatoes. Hence, it would be advisable to take samples from a larger
number of potatoes (a procedure which since has been adopted).

When the samples were tasted in a predetermined order (test No. 4), the tasters distinguished between the samples grown in treated and untreated soil (P. <0.001) but apparently found no difference between the two dosages of insecticide (P>0.9). The response to a given sample apparently was unaffected by the subject's response to the preceding sample. In contrast, when 4 samples from soil treated with 50 pounds per acre and 4 samples from untreated soil were compared (test No. 5, table 5), the distribution of correctly identified samples in the series of 8 might have occurred in 58 out of 10 cases in the absence of any ability to discriminate between treated and untreated samples. The poor quality of both the treated and untreated potatoes and the lack of reference samples may have made the judging more difficult in this case even though all of the tasters were experienced. Although the soil was treated the year before with benzene hexachloride, any off-flavor detected in the potatoes was

TABLE 3.—*Total scores showing extent of off-flavor in potatoes grown during 1947 in soil treated with benzene hexachloride in 1946 at different dosages in pounds per acre*

Test No.	Number of tasters	Total scores at pounds-per-acre dosages of—			
		0	7½	50	50
3	27	−10.61	−6.86	13.99	3.54

TABLE 4.—*Number of subjects reporting off-flavor present and absent in test No. 4, with potatoes grown in soil treated with benzene hexachloride in 1946. The samples were tasted by each subject in the order shown* [1]

Off-flavor rated as	Number of subjects detecting off-flavor with following order of tasting at pounds-per-acre dosages of—			
	0	7½	50	0
Present	6	15	13	6
Absent	14	5	7	14

[1] Treated vs untreated: $X^2=11.25$, $n=1$, $P<0.001$
7½ lb. vs. 50 lb.: $X^2=0.120$, $n=1$, $P>0.9$

TABLE 5.—*Results in palatability test No. 5, consisting of 4 samples of potatoes grown during 1947 in soil treated in 1946 with 50 pounds per acre of benzene hexachloride and 4 samples of potatoes from untreated soil* [1]

Number of samples correctly identified	Frequency	
	Observed	Expected
0		0.3 ⎱5.3
2	6	5.0 ⎰
4	9	11.3
6	7	5.0 ⎱5.3
8		.3 ⎰
Total	22	21.9

[1] Comparing observed and expected frequencies, $X^2=1.106$, $n=2$, $P=0.58$.

like that found in the crop grown the year the soil was treated.   But, some of the tasters reported that the flavor was less marked.

The results of tests on the pure gamma isomer of benzene hexachloride are shown in table 6.   The two samples of potatoes were collected from each of two plots which received the same treatment and from an untreated plot, all in the same field.   As indicated by the significant mean square in the first row of the analysis of variance, the tasters distinguished the treated from the untreated potatoes. Although plots I and II were replicates, the potatoes from one plot tasted more strongly of the insecticide than those from the other.   The tasters described the flavor, when detected correctly, as acid, strong, slightly earthy, or medicinal, terms similar to those used for benzene hexachloride.   Several tasters found the flavor less objectionable than in former tests and others reported no after taste.

<div align="center">CHLORDANE</div>

Although the scores for the different samples in the chlordane test (No. 7, table 7) differed significantly (P<0.05) from one another,

TABLE 6.—*Total scores for 25 subjects showing extent of off-flavor, in duplicate samples from 2 plots of potatoes both grown in soil treated with 0.25 pounds per acre of pure gamma benzene hexachloride, test No. 6, and the accompanying analysis of variance between the scores*

Item	Untreated	Plot I		Plot II	
		Sample 1	Sample	Sample 1	Sample 2
Total scores	−10.13	−3.91	−5.49	10.82	8.77

Source of variation	Degrees of freedom	Mean square	F
Untreated *vs.* treated	1	5.1430	[1] 9.44
Between treated plots	1	8.4042	[1] 15.4
Between samples within plots	2	.0670	
Remainder	96	.5450	

[1] Significant at P<0.01.

TABLE 7.—*Total scores showing extent of off-flavor in potatoes grown in soil treated with chlordane and with chlorinated camphene*

Soil treated with—	Test No.	Number of tasters	Total scores at pounds-per-acre dosages of—				
			0	1	2	4	8
Chlordane	7	28	−4.55	7.84	−8.49	7.34	−2.14
Chlorinated camphene	8	26	−4.22	−7.78	13.11	−1.76	.65
			Total scores at pounds-per-acre dosages of—				
			0	1	1	2	2
Do	9	23	−3.01	−10.65	2.07	11.01	.59

there was no apparent relation between the dose of insecticide and the intensity of off-flavor. Any off-flavor was described as metallic, musty, oily, or chemical. Several remarked that no sample had a strong or objectionable flavor.

CHLORINATED CAMPHENE

Table 7 also gives the total scores for tests Nos. 8 and 9. The relation between dosage and total score was not consistent in either test. Although scores for the different dosage levels differed significantly from one another, the average score for the treated samples did not differ from that for the untreated potatoes. Since the duplicates differed significantly from one another in test No. 9 it is apparent that factors other than the insecticide affected the flavor. The flavor was described as metallic, musty, oily, slightly old, tinny, and earthy. Some found the flavor not objectionable. Chlorinated camphene met with more disfavor, however, than did chlordane.

COMMENTS

Although the results from a single crop-year are not wholly conclusive, they are being reported because of their economic importance.

The commercial dosage for wireworm control for the insecticides tested is 2 pounds per acre. Greenwood[1] reported that benzene hexachloride applied as dust is effective at the rate of 2 to 2.5 pounds per acre when applied broadcast and at the rate of 1.32 pounds per acre as row treatment. Since benzene hexachloride could be detected at a level of 1 pound per acre, and since the flavor is particularly objectionable, its use as an insecticide for control of wireworm in potatoes cannot be recommended.

Further experimentation is needed before definite statements can be made in relation to chlordane. Variations in the chlordane scores indicate that broader sampling would be advisable. Further tests with chlorinated camphene also would be necessary before drawing conclusions as to its effect on the flavor of the potatoes, but it has been found to be ineffective in controlling the wireworm.

SUMMARY AND CONCLUSIONS

Palatability tests were conducted on the presence of an off-flavor in cooked potatoes grown in soil treated with either benzene hexachloride, chlordane, or chlorinated camphene for the control of wireworm.

In the case of benzene hexachloride, the intensity of the flavor increased with the dosage of insecticide and was detected even at the minimum level tested of 1 pound per acre. The flavor was particularly objectionable. Although inconclusive, the evidence suggests that potatoes grown in soil treated in the preceding year carried the same flavor. Apparently the pure gamma isomer of benzene hexachloride also imparted a foreign flavor to potatoes but the flavor was less objectionable than with the crude form.

Tests with potatoes grown in chlordane-treated soil were inconclusive but any resulting flavor from chlordane was much less objectionable than the flavor of benzene hexachloride.

The evidence for chlorinated camphene also was inconclusive. However, its flavor seemed to be more objectionable than that of chlordane.

THE DIGESTIBILITY AND METABOLIZABILITY BY LAMBS OF A STANDARD RATION OF ALFALFA AND CORN AND ONE CONTAINING COTTONSEED HULLS [1]

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INTRODUCTION

In a large section of this country cottonseed hulls are fed extensively to cattle and sheep. Since hulls are extremely low in protein and are relatively unpalatable, cottonseed meal is frequently fed with them. Such rations may be adequate in protein and in energy, but will be deficient in calcium, carotene, and vitamin D.

Studies reported in this paper were made in the course of investigations whose object was to formulate nutritionally adequate rations containing significant quantities of cottonseed hulls and to compare them with a standard ration of alfalfa hay and corn, using sheep as experimental subjects.

REVIEW OF THE LITERATURE

The first reports dealing with the digestibility of cottonseed hulls were made by Harrington (10) [2] in 1891 and Emery and Kilgore (2). The Texas investigator (10) found that five steers digested 41.6, 5.7, 78.0, 30.4, and 41.2 percent respectively, of the dry matter protein, either extract, nitrogen-free extract and fiber. The North Carolina report (2) states that a cow in late lactation was given 21 pounds of cottonseed hulls daily but never completely consumed this amount. From a 4-day collection of feces digestion coefficients were calculated as follows: Dry matter 35.9, protein 24.6, ether extract 80.6, nitrogen-free extract 40.3, and crude fiber 27.1 percent. These early reports indicate that cottonseed hulls constitute an extremely low-grade feed.

Results of further investigations at the same institutions by Fraps (9) and by Emery and Kilgore (3), involving a cow, two goats, and five sheep, differed from those reported previously in that digestibility of protein was a negative value in all cases but one. The apparent digestibility of protein by a goat amounted to 1.2 percent.

The results obtained from these investigations represent the available data on the direct determination of digestibility of cottonseed

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[2] Italic numbers in parentheses refer to Literature Cited, p. 488.

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hulls. The Texas and North Carolina stations have also computed digestibility by the indirect method: Fraps (7, 8, 9), Emery and Kilgore (2, 3, 4). One cow, 10 sheep and 9 steers were used in the experiments from which the computations were made. There was a consistently negative protein digestibility (12–61 percent) when the hulls were fed with alfalfa hay, with cottonseed meal and alfalfa hay, or with cottonseed meal alone. The average total digestible nutrients of cottonseed hulls as calculated by Schneider (*11*) from the data referred to above are as follows: For cattle 45.2 percent, and for sheep 52.8 percent on the dry basis. The digestible protein content of the hulls averages −0.6 percent, according to the same author.

EXPERIMENTAL PROCEDURE

In view of the obvious effect of associative digestibility exhibited by cottonseed hulls in earlier work, an effort was made to determine the digestibility and metabolizability of complete rations only.

The experimental animals were five wether-lambs bred on the experiment station farm. Throughout the experiment (fall of 1947) they were maintained in metabolism crates. The composition of rations fed is shown in table 1. Ration 1 was composed of 44.5 percent of good-quality chopped alfalfa hay and 55.5 percent of yellow corn. Ration 2 was composed of 50.6 percent of yellow corn, 15.5 percent of cottonseed meal, 4.0 percent of alfalfa leaf meal, and 29.9 percent of cottonseed hulls containing 10 percent of molasses. The rations were so compounded as to be nearly equal in gross nutrient content.

Sufficient feed was mixed and weighed in individual portions before each trial to last throughout a 10-day preliminary period and an 8-day collection period. During the latter period feces and urine were collected daily. Feces were preserved by drying the total daily samples, separately. At the end of the trial these samples were mixed, ground, and sampled carefully for analysis. An aliquot of each day's urine collection was preserved with acid and refrigeration. These daily aliquots were mixed and sampled for nitrogen and energy determinations at the end of the experimental period. Analyses of feed and feces were made by the usual procedures. The urinary nitrogen was determined by the Kjeldahl method and the urine energy by a modification of methods used at the Illinois and Pennsylvania stations.

Metabolizable energy of the rations was calculated from data obtained as indicated in the preceding paragraph and by using the equation, $E=2.41X+9.80$, to calculate the methane produced by fermentation; see Swift et al. (*13*). Correction for nonmetabolizable portions of protein gain was also made.

TABLE 1.—*Composition of rations fed to lambs (air-dry basis)*

Ration No.	Dry matter	Protein	Fat	Fiber	Nitrogen-free extract	Ash	Energy
							Calories per
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*kilogram*
1 -------------------------	89.7	12.3	3.09	13.7	57.7	2.9	4,081
2-------------------------	91.5	12.5	2.60	15.5	58.2	2.7	4,101

Since a detailed description of the method used in determining the energy content of the urine was not found in the recent literature, a convenient technique is described.

The urine aliquot, preserved with acid, is neutralized with alkali. Fifteen ml. of this aliquot are placed in an evaporating dish and dried at 60° C. until a thick syrup is formed. Then with the aid of hot distilled water and a stirring rod the sample is transferred to a combustion cup containing a weighed amount of cellulose block. This material is redried and a second washing is made from the evaporating dish to assure complete transfer of the urine. At this time the block is distributed over the cup by shredding with a pointed glass rod. After a final drying the sample is ready for combustion in the calorimeter.

The gross energy content of the cellulose used must of course be determined by separate analyses and this value subtracted from the total heat evolved. Since some nitrogen will be lost in the course of drying, samples must be prepared in the same way as for the energy determination, and the loss of nitrogen by drying determined by the Kjeldahl method. If it is assumed that the nitrogen lost originated from urea, each gram of nitrogen lost will represent a loss of 5.424 calories. Under the conditions of the experiment this loss of energy in drying amounted to from 2 to 4 percent of the gross energy of the urine.

RESULTS AND DISCUSSION

Table 2 presents a summary of the data obtained. All differences observed between the two rations are statistically significant at the 1-percent level except those for fiber digestibility.

From the data given in table 2 it may be determined that the lambs on the supplemented ration digested 91 percent as much dry matter, 82 percent as much protein, 117 percent as much fat, 92 percent as much nitrogen-free extract, and 93 percent as much energy as on the alfalfa hay-corn ration. Sixteen percent of the digestible energy of the alfalfa hay-corn ration and 15 percent of that of the supplemented

TABLE 2.—*Percentage digestibility of rations* [1] *for lambs, and metabolizable energy expressed in calories per kilogram and as percent of the gross energy*

Lamb No.	Ration No.	Dry matter	Protein	Fat	Fiber	Nitrogen-free extract	Energy	Metabolizable energy	Energy
								Calories per kilogram	Percentage of gross
1	1	79.3	77.0	72.7	58.2	87.6	77.8	2,981	65.5
	2	72.4	61.7	87.1	52.2	81.3	71.7	2,788	60.9
5	1	77.8	72.8	74.0	56.3	86.5	76.3	2,933	64.3
	2	70.2	58.6	86.3	50.1	79.5	69.4	2,711	59.1
6	1	79.7	78.5	72.0	55.8	88.4	78.4	3,008	66.0
	2	72.0	67.3	85.2	56.0	77.7	71.0	2,760	60.3
7	1	76.5	73.4	68.2	53.8	84.9	74.6	2,850	62.6
	2	71.8	60.7	85.9	53.8	80.4	70.8	2,743	59.8
9	1	78.4	75.5	74.6	50.4	86.2	76.9	2,979	65.5
	2	72.1	60.5	78.9	51.2	81.9	70.7	2,744	59.8
Average	1	78.3	75.4	72.3	54.9	86.7	76.8	2,950	64.8
	2	71.7	61.8	84.7	52.7	80.2	70.7	2,749	60.0

[1] As explained in connection with reference to table 1, ration 1 was composed of 44.5 percent of good-quality chopped alfalfa and 55.5 percent of yellow corn. Ration 2 was composed of 50.6 percent of yellow corn, 15.5 percent of cottonseed meal, 4.0 percent of alfalfa leaf meal, and 29.9 percent of cottonseed hulls containing 10 percent of molasses.

hull ration were lost to the animals by way of urine and fermentation in the gastro-intestinal tract. Although of similar gross energy content, the supplemented hull ration contained only 93 percent as much metabolizable energy as the alfalfa hay-corn ration. This difference was due to the lower digestibility of the former ration.

In view of the similarity of composition of the rations, as judged by the usual feed analyses, it was evident that some factor other than those shown by analysis was affecting the digestibility of the rations. Lignin analyses, made by the 72 percent H_2SO_4 method as modified by Ellis, Matrone, and Maynard (*1*) gave a possible clue. The lignin content of the alfalfa hay-corn ration was 4.40 percent and of the supplemented hull ration 9.45 percent, on the dry basis. Previous studies at this station by Forbes and Garrigus (*6*) have shown a close correlation between lignin content and digestibility of pasture forages. The present investigation also indicates a possible influence of lignin on the digestibility of mixed rations.

The common explanation for the inhibiting effect of lignin on digestibility is that the lignin forms a mechanical barrier to the action of digestive juices. Such an explanation, however, does not explain all the observations of this study. For example, although a very minor portion of the total protein of the supplemented hull ration was contained in the highly lignified hulls, the lignin apparently lowered the total protein digestibility by 18 percent. The supplemented hull ration was more compact than the alfalfa hay-corn ration, although the hay in the latter ration was chopped into about 1-inch lengths. It is possible that rumination by animals receiving the more compact ration was less extensive. Such a finding has been made in studies with dairy heifers by Swanson and Ragsdale (*12*), although they found no difference in digestibility between chopped and finely ground hay. The compactness of the supplemented hull ration might be a factor in causing the difference in digestibility between the two rations. Another factor might be the presence in cottonseed hulls of some substance inhibiting the normal action of the rumen microflora or the digestive enzymes. Wise (*14*), in summarizing the action of bacteria on lignin, notes that isolated lignin is sometime inhibitory to bacterial growth. However, a similar effect of lignin "in situ" has not been proven and specific data on this subject, must await further investigation.

The data here presented also afford evidence that under some circumstances the material analyzed as lignin by the method used in this study may be digested appreciably by ruminants. Ninety-seven percent of the lignin fed in the alfalfa hay-corn ration was recovered as compared with 85.7 percent when the supplemented hull ration was fed. It is possible that some of the resins and pentosans present in cottonseed hulls are not affected by the reagents used in the lignin analysis but are subject to some degree of change in the digestive tract of the sheep.

The average daily nitrogen balance of the lambs on the alfalfa hay-corn ration was 2.86 grams (range 1.51 to 4.03) and that on the supplemented hull ration 2.59 grams (range 0.57 to 3.74). These values are not significantly different.

Carotene analyses were not made but calculations based on average published analyses showed that the rations might be expected to contain an adequate amount of this nutrient. The supplemented hull ration contained only 70 percent of the recommended allowance of calcium. This deficiency would not be likely to affect the results of an experiment of this type.

In view of the desirability of expressing the nutritive value of rations in terms of metabolizable energy, it is of interest to compare the results obtained in this experiment with those calculated from digestible nutrients by the method of Axelsson, cited by Forbes and Thacker (*5*). In this computation the following factors were used per gram of digestible nutrient: Protein 4.4, fat 8.0, fiber 2.9, nitrogen-free extract 3.7. Table 3 shows the estimates of metabolizable energy obtained by the method of Axelsson (calculated) and in this experiment (experimental).

Axelsson's factors were derived for use in determining the metabolizable energy of rations for cattle, but the data presented herewith show that these same factors may also be useful with rations for sheep.

TABLE 3.—*Comparison of methods of calculating metabolizable energy of rations for sheep*

| Lamb No. | Metabolizable energy (calories per kilogram of feed) | | | |
| | Alfalfa hay-corn ration | | Supplemented hulls ration | |
	Calculated	Experimental	Calculated	Experimental
1	3,006	2,981	2,738	2,788
5	2,948	3,008	2,663	2,711
6	3,019	2,850	2,700	2,760
7	2,887	2,979	2,713	2,743
9	2,940	2,950	2,719	2,744
Average	2,960	2,950	2,707	2,749

SUMMARY

The digestibility and metabolizability of two rations by sheep are reported. Ration 1 was composed of alfalfa hay and corn, while ration 2, similar in gross nutrient analysis, was composed of corn, cottonseed meal, alfalfa leaf meal, cottonseed hulls, and molasses.

The lambs fed ration 2 digested 91 percent as much dry matter, 82 percent as much protein, 117 percent as much fat, 92 percent as much nitrogen-free extract, and 93 percent as much energy as did those fed ration 1. Of the digestible energy of both rations 85 percent was metabolizable.

The metabolizable energy of the rations as calculated from this experiment was in close agreement with that calculated by the method of Axelsson.

A convenient method for determining the gross energy value of the urine is described.

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STABILITY OF CAROTENOIDS IN GROUND
DEHYDRATED CARROTS [1]

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INTRODUCTION

The possibility that antioxidants might protect the carotene pigments in dehydrated carrots was suggested by the observation (Weier, 8) that these pigments dissolved in intracellular oil droplets when small pieces of carrot root were dried. Upon storage the pigments disappeared from the oil droplets whereupon they gave a positive test with Schiff's reagent, indicating the occurrence of oxidative rancidity in the oil. It seemed possible that the addition of an antioxidant to the oil droplets would retard pigment degradation in dehydrated carrots.

Since in blanched, but undried carrot dice, as much as 90 percent of the pigment may disappear in 24 hours, the first tests were carried out on this type of material (*9, 10*).[2] Positive results were obtained: ascorbic acid, nordihydroguaiaretic acid, oxalic acid, and phosphate buffer at neutrality greatly retarded pigment breakdown. That antioxidants will also protect the pigment in dehydrated carrots has been shown in some preliminary tests (*11*).

Lovern (*4*) and his associates studied the influence of antioxidants on the shelf life of dehydrated carrots. They found that under certain conditions, the pigment in ground dehydrated carrots received a slight amount of protection while that in dehydrated dice did not.

The problem of protecting the carotenoid pigments in dried carrots is complicated by a number of physical factors that are not present in experiments on pure pigments in pure solvents. Some of these factors are the following:

(1) The concentration of the pigment and the nature of the oil in which the carotenoids are dissolved is not known.

(2) The particulate nature of the oil-pigment complex presents an enormous surface which is unquestionably a factor in the oxidation of the pigment. Both the area and chemical nature of this surface are of importance.

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(3) The cellular structure of the carrot root, varying in different regions of the root, must influence the rate of diffusion of the oxygen and consequently the rate of oxidation of oil and pigment.

(4) Carbohydrate content, particularly starch, which becomes gelatinized during blanching, may be a factor in limiting the rate of pigment degradation.

(5) The total amount of pigment present in the dried dice is likely to influence the rate of breakdown. This factor may not be, however, directly related to the mass action law. The concentration of the pigment in the oil droplets may be uniform but there may be variations in the number of oil droplets per unit volume. This would result in a great increase in surface with an increased rate of pigment breakdown.

6) Natural antioxidants such as tocopherols are known to be present in carrots and they appear to have effect in protecting the pigment when it is concentrated in a natural oil extract. The relationship of such natural antioxidants and the oil and pigments in raw and dehydrated carrots is not known.

(7) Season of harvest and preprocessing handling may influence the stability of the pigment.

Although the main interest of these investigations as indicated was the eventual testing of the influence of certain antioxidants on the rate of breakdown of the carotenoid pigments, certain details regarding the breakdown of the pigments in blanched dehydrated carrots under accelerated storage must first be ascertained. This paper discusses the variation in the pigment concentration in carrot roots during growth and the localization of the pigment within the root. Rates of breakdown in dehydrated carrots under accelerated storage conditions are reported for the total pigment complex, for alpha and beta carotenes, and for the more strongly adsorbed carotenoid components. The influence of temperature and oxygen concentration on the rate of pigment degradation is also discussed.

MATERIAL AND METHODS

PREPARATION AND STORAGE

Imperator carrots (Ferry-Morse seed) were used in these studies. Unless otherwise stated all carrots used were grown in Davis and harvested immediately before needed.

Diced carrots were prepared for the storage experiments by blanching in steam for 5 minutes, after which they were dehydrated at 140° F. for 8 to 10 hours to 6 to 7 percent moisture, by the 40-hour vacuum oven method, in an experimental dehydrator built by the Agricultural Engineering Division of the University of California. After drying, the dice to be used in the accelerated storage tests were ground to pass a 20-mesh sieve. In order to insure uniform particle surface, the fine particles were sifted through a 48-mesh sieve and discarded. Five-gram samples of these ground carrots were stored in stoppered 300-ml. Erlenmeyer flasks which were opened at regular intervals. When larger amounts of ground carrots were stored, unless otherwise stated, atmospheric oxygen was available to the sample.

PIGMENT DETERMINATION

For total pigment determinations 0.1-gram aliquots of the dehydrated material were rehydrated by pouring boiling water over them and allowing them to stand for about 30 minutes. The water was decanted off and the pigments were completely extracted in acetone and made up to 100-ml. volume. The optical density of this solution was measured on a Lumetron Colorimeter using a 440-mμ filter. Since in the majority of these tests the pigments were not separated it seemed unwise to express the results as milligrams of beta carotene.

All extracts were handled in low actinic red glassware and when measurements were not taken immediately after preparation of the extract it was kept at 4° C. All measurements were made within 24 hours of the preparation of the extract.

In connection with storage and antioxidant investigations of dehydrated carrots, it is convenient to use carotenoid degradation rates in determining deterioration of quality. In its simplest form such a procedure involves the extraction of the total pigment with acetone and the determination of the total optical density of this crude extract with a photoelectric colorimeter, as just described. Carrots, however, contain a series of polyene compounds. Consequently, in a detailed investigation of storage changes it is important to ascertain the relative stability of at least the major members of this series and to determine whether there is any selective antioxidant effect upon them.

The approach to this aspect of the problem was divided into two parts, (*1*) a study of pigment changes with growth of the carrots, and (*2*), a study of the stability of individual pigment components in dehydrated carrots under accelerated conditions of storage and subject to antioxidant treatments.

Samples were taken for chromatographic analyses from the regularly prepared and stored material. One-gram portions were rehydrated, drained, extracted with acetone, transferred to petroleum ether and washed three times with water. They were then dried with anhydrous sodium sulphate and evaporated under vacuum at room temperature in the dark to about 1- to 2-ml. volume in most cases.

The chromatographs were prepared on 1 : 1 magnesia [3] Super-Cel columns (13-mm. diameter) using petroleum ether as a pigment solvent. Petroleum ether with 4 percent acetone was used for the development. About three-fourths of an inch of anhydrous sodium sulphate was placed at the top of the column to insure complete removal of all moisture before development.

The columns were protected from the light and adequate precautions were taken to prevent drying. After the development the individual components were either collected as liquid fractions below the column or the column was pushed out and immediately divided and the individual bands placed in acetone. In the latter case (the usual procedure) the adsorbent was removed from the pigment fractions by filtering through fine sintered glass filters and washing with acetone. Thus optically clear solutions of the individual fractions were obtained.

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[3] Adsorptive powdered magnesia No. 2641, Westvaco Chlorine Products, Newark, Calif.

In general only three groups of pigments were separated: (1) Alpha carotene, (2) beta carotene, (3) the balance of the carotenoids called group "A" for convenience. In some runs zeta carotene (which is adsorbed just above beta carotene) was also determined. After preparation and proper dilution of the samples the absorption of light by each was measured, using a photoelectric colorimeter and a 400-mμ filter. The results are reported as relative optical density calculated on the basis of the pigment in 1 gram of dehydrated carrot dissolved in 100 ml. of solution.

Losses in transferring to petroleum ether and in chromatographing were determined and recovery was usually more than 90 percent based on photoelectric determinations using the 440-mμ filter. Table 1 shows a typical set of analyses with losses in total pigment incurred in the process.

Losses in individual fractions were also checked to see whether there were significantly greater losses in one fraction than in another. The alpha and beta carotene fractions were rechromatographed to determine whether the percentage lost by either fraction was significantly greater than the total loss for the combined fractions. Table 2 shows that in both cases approximately the same percentage of loss was obtained as when total pigment was used.

TABLE 1.—*Percentage recovery of total carotenoid pigment after chromatographing from sample of blanched, dehydrated, ground carrots stored at 40° C.*

[Figures are optical density determinations made with a photoelectric colorimeter using a 440-mμ filter]

Item measured	July 25	July 29	Aug. 1	Aug. 5	Aug. 8
Optical density before chromatographing	3 645	3. 630	3. 655	3 380	3 080
Optical density after chromatographing	3. 422	3. 512	3. 307	3. 190	2 960
Percent recovered	93 8	96 8	92 2	93. 4	96. 0

TABLE 2.—*Losses in individual carotenoid pigments during rechromatographing*

[Figures are in relative optical densities determined using a 440-mμ filter and a photoelectric colorimeter]

Fraction	Before rechromatographing	After rechromatographing	Percent lost in rechromatographing
Oct. 19·			
Alpha carotene	1. 487	1. 399	5. 9
Beta carotene	2. 617	2. 438	6. 8
Nov. 28:			
Alpha carotene	. 347	. 313	9. 8
Beta carotene	. 335	. 310	7. 5
Group "A"	. 177	. 165	7. 0

PIGMENT CHANGES WITH GROWTH OF CARROTS

It has been reported (*5*) that there is no marked variation in the ratio of alpha to beta carotene with growth of carrots during a period of from 9 to 26 weeks after germination, although the total carotene content increased about 25 times during this period. A further test of this point, particularly during the early stage of growth, was made in the present study. Changing concentrations in the various carotenoid fractions from the third to the ninth week of carrot growth are

shown in figure 1.   It should be noted that the results reported are optical densities obtained using a 440-mμ filter and are not absolute values of carotenoid concentration.

Because of the lack of complete uniformity of germination and growth the carotenoid concentrations per unit fresh weight of carrot are plotted against the average weight of individual carrots rather than age.   The last points on the graph show the pigment content of 9-week-old carrots whose average weight was 10.8 gm. per carrot.   Carrots of uniform size were selected for each set of determinations.   The first determinations were made when the seedlings were from 2 to 8 cm. in length and averaged 0.066 gm. fresh weight (90 percent water).   Two hundred and fifty-nine carrots were used for the analysis.   The last determinations were made on carrots 15 cm. long and 10.8 gm. in weight.   Only seven of these carrots were used in the analysis.
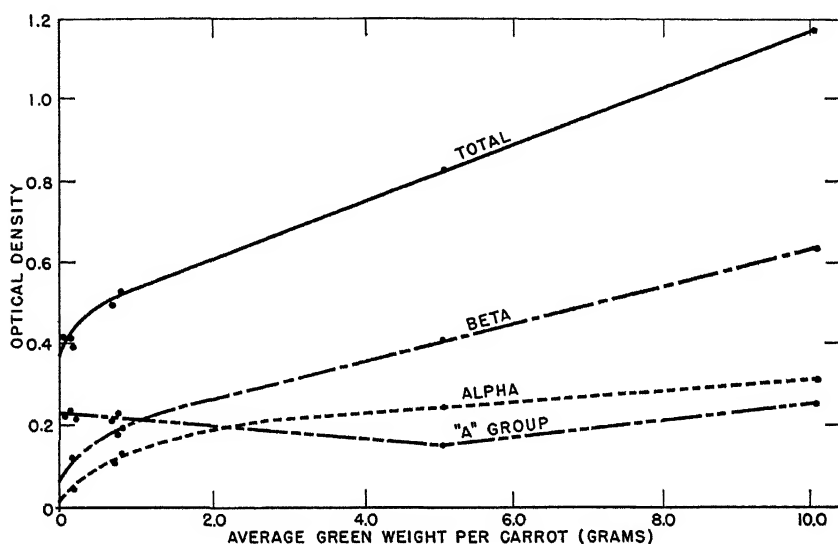
FIGURE 1.—Changes in carotenoid pigments in carrot roots during growth.   Densities based on 10 gm. of fresh carrot per 100 ml. of solution.   (Group "A" includes all carotenoid pigments more strongly adsorbed than beta carotene.)

The results show that the very young seedlings contain less alpha and beta carotene than the remainder of the carotenoid pigments—chiefly strongly adsorbed carotenols.   During the development of the carrot the concentration of the carotenols within the tissue remains relatively constant while there is a uniformly rapid increase in alpha and beta carotene.   There is some indication that beta carotene is present in a slightly greater proportion in the young carrots than in the older ones, i. e., the ratio of beta to alpha carotene dropped from 3.5 to 1.6 and then increased to slightly over 2 which was about the ratio found in the mature carrots of these plantings.   The ratio of the beta and alpha carotene to the balance of pigments increased from 0.61 in the first sample to 3.8 in the 9-week-old carrots.   In mature carrots of this strain this ratio is between 8 and 9.

These data would indicate that oxidized carotenes, or perhaps colorless polyenes which were not determined, are the major polyene components in carrot seed, and that the alpha and beta carotenes are formed chiefly upon germination and growth of the seedling, being laid down in the manner of a waste product, and are at no time drawn upon to any appreciable extent as a reserve. On the other hand, the carotenols appear to be of fairly uniform concentration during the development of the carrots and presumably occur in high concentration in the seed. This is an agreement with conditions found by Strain (7) for barley seeds. In this latter case the principal constituents of seeds were the xanthophylls, carotenols with large amounts of fluorescent colorless materials and small amounts of beta carotene being present. Unfortunately, limitations of facilities prevented the determination of the colorless polyenes such as phytofluene.

The population of the plantings of Imperator carrots used in this study showed a fairly wide range of color. Chromatographs showed that in general the concentrations of alpha and beta carotenes and consequently of the total pigment concentration were considerably increased as the depth of color increased in carrots of the same age in any planting. The lightest colored carrots in any planting had the greatest proportion and the greatest absolute amount of carotenoids other than alpha and beta carotenes. In the storage experiments described later, the light-colored carrots were not used.

Harper and Zscheile (2) have reported that several varieties of carrots have a greater proportion of carotenols in the xylem than in the phloem, which had a predominant amount of beta carotene. Certain of the storage experiments in the present investigation involved the use of phloem and xylem tissues separately. The summary of the results of chromatographic separation of the pigments in composite samples of these two issues, shown in table 3, agrees with the general conclusions of Harper and Zscheile.

TABLE 3.—*Contribution by the various carotenoid fractions to the total optical density of pigment extracts of phloem and xylem tissue in imperator winter-grown carrots*

| Sample No. | Percent of total optical density | | | Total optical density |
	Alpha carotene	Beta carotene	"A" group	
1.—Phloem	19.4	73.3	7.3	2.970
2.—Phloem	19.0	73.3	7.7	3.210
3.—Phloem	21.1	72.1	6.8	2.970
4.—Xylem	14.4	66.0	19.6	1.137
5.—Xylem	16.4	67.2	16.4	1.092

RATE OF PIGMENT BREAKDOWN UNDER ACCELERATED STORAGE

STORAGE IN AIR

TOTAL PIGMENT DEGRADATION

The dissolution of the carotenes in the oil droplets and the positive Schiff tests given by these droplets after the degradation of the pigment, suggested that the rate of pigment breakdown might follow a

typical fat-oxidation curve, and that an initial induction period, followed by a rapid rate of oxidation might be expected. With but few exceptions this type of breakdown was observed: There was an induction period of from 3 to 5 days followed by a period of increased rate of pigment breakdown, which was generally maintained until from 50 percent to 60 percent of the pigment was degraded. The points along this portion of the curve, in all experiments, fell on a straight line when plotted on standard coordinate paper (fig. 2). Regression lines were run for this portion of the curve for many experiments. The fit was unusually good, the standard error being between 2 and 4 percent.

After the decomposition of 50 percent to 60 percent of the pigment, the rate of breakdown slowed greatly. This occurred in many experiments, regardless of treatment, in such a manner that the residual pigment content after long storage times—from 30 to 40 days in experiments with ground carrots—was approximately the same.
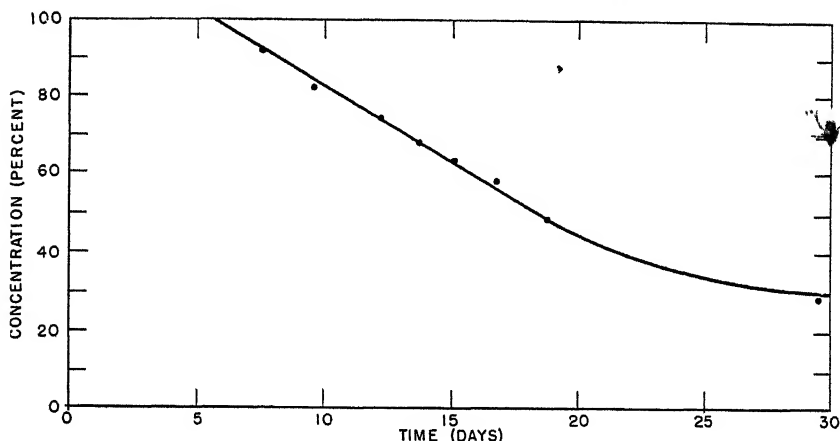

FIGURE 2.—Rate of carotenoid degradation in blanched, dehydrated, ground carrots stored in air at 40° C.

Since the main interest was to extend the induction period and to greatly retard the rate of breakdown during the straight-line portion of the process no attention was paid to the latter stages of breakdown.

Because of the shape of the breakdown curve it is of importance, when making comparisons between various experiments, to be certain that the same portions of the curve are being compared.

The series of experiments herein reported extended over a period of 2 years. In analyzing the data obtained it was necessary to know the extent of the variations (1) in tests run at the same time on separate lots of carrots from one or more plantings, and (2) in tests run at different times on lots of carrots from different plantings.

In order to test the first point (the second will be discussed in a subsequent section) six series of tests were run on each of two plantings of carrots. The roots in one planting were 6 months of age, those in the second 3 months old. The results shown in figure 3 are those obtained from the younger roots. The older roots yielded figures
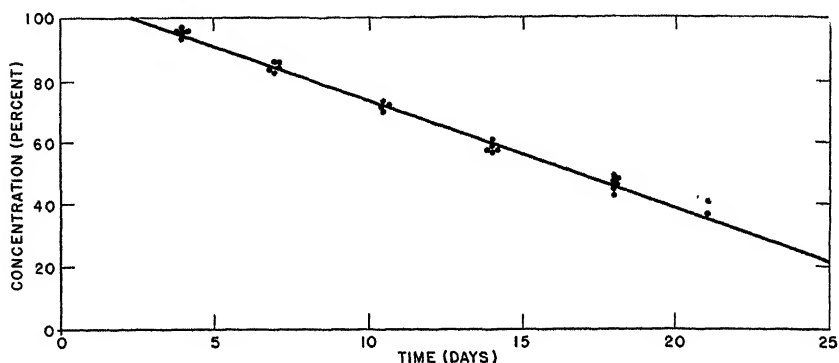
FIGURE 3.—Rate of carotenoid degradation in six samples of blanched dehydrated ground carrots stored in air at 40° C.

which were so similar as to be difficult to include in a single small graph together with the result of the younger roots. The curve shown is a standard regression line for the points involved. The standard error is 2.2 percent. Other significant data are shown in table 4.

TABLE 4.—*Typical carotenoid breakdown in ground dehydrated carrots stored at 40° C.*

Age of roots	Average carotenoid content in gamma of Eastman carotene per gram of dried carrots	Initial optical density reading	Percent breakdown per day	Percent breakdown 20 days	Standard error (percent)
3 months	710	0. 240	3.11	55	2. 2
6 months	1, 260	. 420	3. 49	57	3. 2

The stored samples of ground dehydrated carrots gradually lost weight during storage. A 5-gram sample of ground dried carrots under the storage conditions described lost approximately 0.26 gm. (5 percent) in 40 days' storage. During 20 days' storage, the normal duration of the majority of experiments, the weight lost was 0.07 gm. Carotenoid concentrations calculated for the 20-day storage period should therefore be decreased slightly. The error is small and reasonably uniform in all experiments. No corrections for it have been made.

DEGRADATION OF INDIVIDUAL PIGMENTS

The characteristic curve representing pigment degradation in blanched ground carrots stored at 40° C. in air-tight containers is shown in figure 4. In this case no induction period was observed. This graph shows the contribution of alpha and beta carotenes and the more strongly adsorbed carotenoids to the total pigment breakdown curve. It should be emphasized again that the results represent only the optical density determined by use of the photoelectric colorimeter using the 440-mμ filter and should not be interpreted as representing
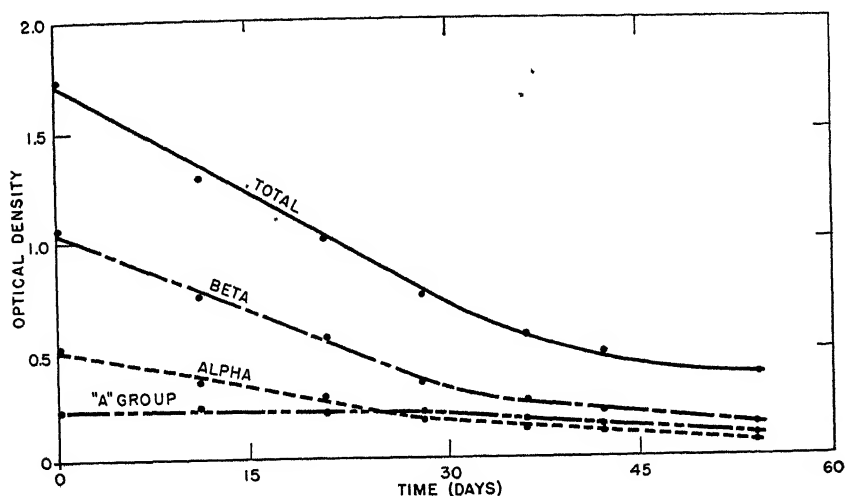
FIGURE 4.—Rate of degration of individual carotenoid pigments during storage of blanched, dehydrated, ground carrots at 40° C. in air. Optical densities based on 1 gram of dehydrated carrots per 200 ml. of solution. (Group "A" refers to all carotenoids more strongly adsorbed than beta carotene).

the actual concentration of the various pigment fractions. However, the values do represent the contribution of the individual components to the total optical density determined with the colorimeter at this wave length. It will be noted that the alpha and beta carotene fractions both have curves of the same general shape, and because they constitute the major portions of the pigment present, the total pigment degradation curve is of the same shape. Mackinney and Fratzke (6) have also observed that alpha and beta carotenes oxidized at about the same rate during storage of dehydrated carrots.

The more strongly adsorbed pigments (zeta, gamma carotenes, and the carotenols) show a fairly constant density throughout the storage period with a slow decrease appearing only when the rate of breakdown of alpha and beta carotene began to level off. This might indicate that degradation products of alpha and beta carotenes contribute to the density of the more strongly adsorbed group. A further indication that this is the case may be found in figure 6 which shows a small but definite increase in this group during the early storage period when there was a rapid breakdown of alpha and beta carotenes. This increase has been observed in a number of experiments.

GAS STORAGE

Hoffman, Lum, and Pitman (3) have studied the relation between rate of carotene degradation in dehydrated alfalfa and oxygen tension. They concluded "that at least the initial rates of carotene destruction are very nearly proportional to the percentages of oxygen in the storage." When oxygen tension varied slightly or not at all, i. e., when the gas mixture containing 3 percent oxygen was renewed daily, the rate of carotene breakdown was directly proportional to time. In their experiments the dried alfalfa meal occupied approximately 60

percent of the storage container, which was a Schwartz U-tube of about 26-cc. capacity.

In the experiments with ground dehydrated carrots, herein reported, the 5-gm. sample in a 300-ml. flask occupied about 1 percent of the volume of the container. The air in the container was renewed every 3 or 4 days. Although gas analysis of the atmosphere within the containers was not made the conditions of the experiment were such that variations of oxygen tension within the storage containers were not a factor in determining the course of the reaction.

Only one series of comparisons between O_2, air, and CO_2 was run (fig. 5). Some breakdown does occur in CO_2, the nature of which was not determined. A comparison of the curves for air and O_2 is of interest. The curve for rate of breakdown in air is, as usual, comprised of an induction period plus a straight line portion of rapid breakdown.
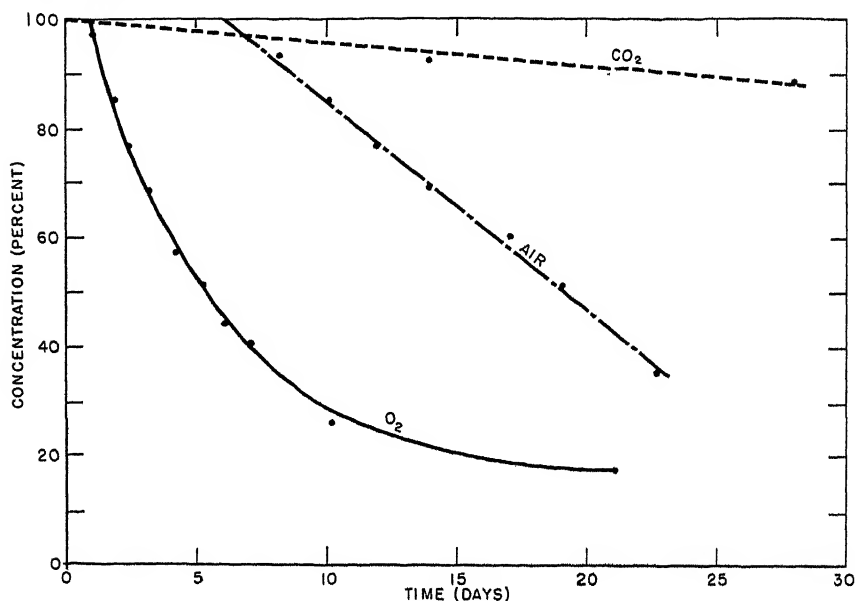
FIGURE 5.—Influence of storage atmosphere on carotenoid degradation in blanched, dehydrated, ground carrots stored at 40° C.

In oxygen the induction period is shorter and a true straight line period of breakdown appears to be absent. Instead the points lie along an exponential curve. When these points are plotted on semilog paper a straight line results. This indicates that the lower oxygen tension of the air is a modifying factor in the pigment breakdown. With a great excess of oxygen the points obtained suggest that the rate of pigment degradation is more closely proportional to the amount of pigment present.

In spite of the straight line obtained for breakdown in air, pigment concentration, at least at the start of the experiment, influences the rate of pigment breakdown.

A further study in which pigment breakdown was accelerated by storing the ground carrots in an atmosphere of oxygen showed that

alpha and beta carotenes under these conditions undergo oxidative decomposition at about the same rate. The ratio of beta to alpha carotene was 3.61 at the start of the experiment, increased to 4.14 at 5 days, and was 3.11 at the end of 27 days when 90 percent of the pigment was destroyed. At the same time there was, however, a slight increase in the density of the strongly adsorbed pigments followed by a decrease (fig. 6).
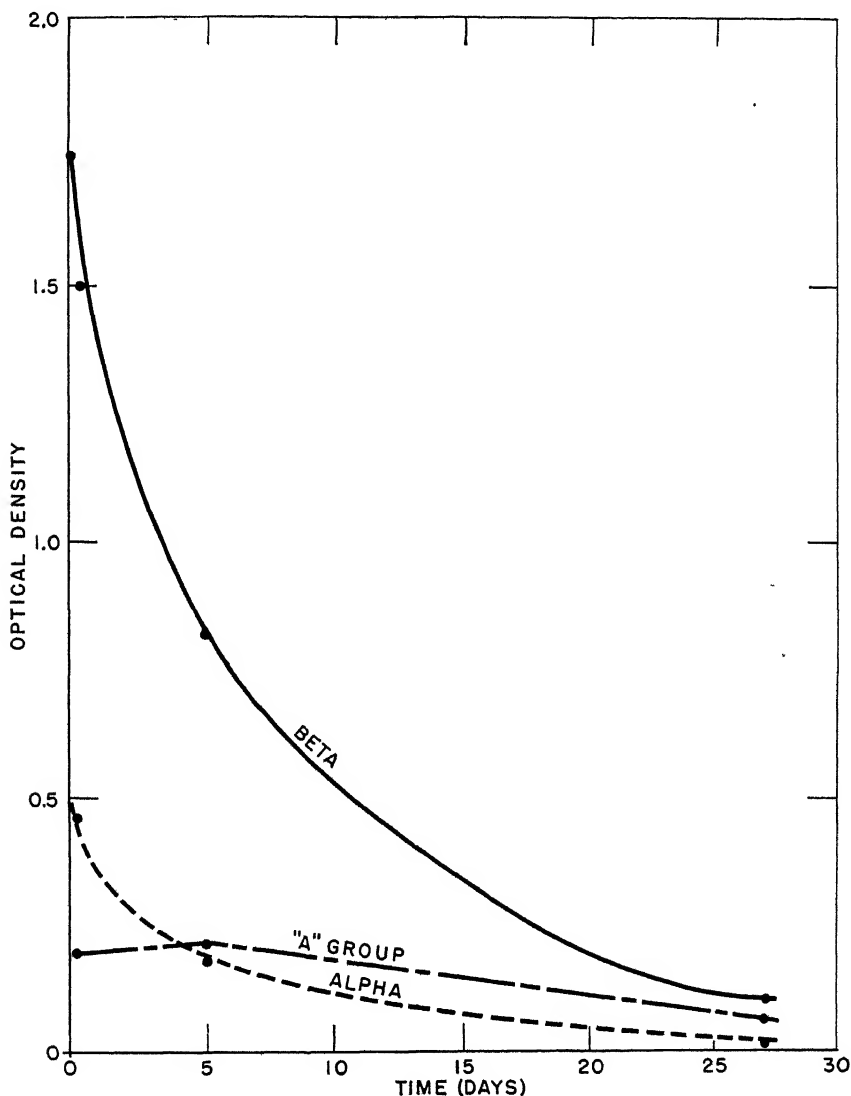

FIGURE 6.—Carotenoid degradation in blanched, dehydrated, ground carrots stored at 40° C. in oxygen. (Group "A" refers to all carotenoids more strongly adsorbed than beta carotene.) Densities based on 1 gram of dehydrated carrots per 200 ml. of solution.

EFFECT OF TEMPERATURE

One difficulty encountered in using accelerated breakdown tests is that of establishing that the accelerated reaction is following a course that is similar to the slower reaction, about which information is desired. It has, for instance, been shown that rapid fat oxidation at elevated temperatures is not strictly comparable to oxidation at lower temperatures (1). In order to determine the best temperature for studies on carotene degradation in samples of dehydrated carrots, pigment breakdown was followed at 30°, 40°, 50° and 60° C. It was found that the Q_{10} [4] between 30° and 40° and between 40° and 50° C. remained constant at approximately 1.60 during the straight line period of carotene breakdown. The ratio between breakdown at 50° and 60° C. is not constant, decreasing gradually from 1.48 to 1.24 (table 5 and fig. 7). Forty degrees centigrade was selected as the
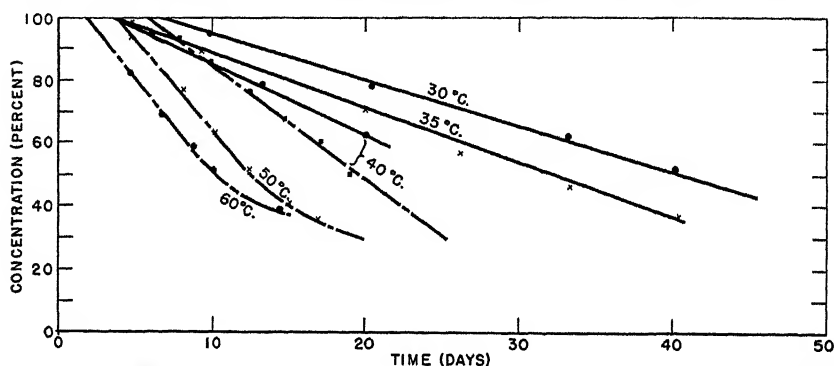
FIGURE 7.—Effect of storage temperature on carotenoid degradation in blanched, dehydrated, ground carrots stored in air.

experimental storage temperature with good assurance that the course of pigment breakdown at both higher and lower temperatures was comparable with that found at 40° C.

TABLE 5.—*Comparison of pigment breakdown at different temperatures*

| Percent of pigment degraded | Time in days for pigment degradation in— | | | | | Q_{10} | | |
| | Experiment No. 1, at— | | Experiment No. 2, at— | | | | | |
	30° C.	40° C.	40° C.	50° C.	60° C.	30 to 40° C.	40 to 50° C.	50 to 60° C.
10	4.0	7.8	8.4	5.5	3.7	1.80	1.52	1.48
20	20.2	12.0	11.0	7.1	5.3	1.67	1.60	1.33
30	26.8	16.2	13.8	8.7	6.7	1.58	1.58	1.29
40	35.2	20.6	16.5	10.3	8.3	1.65	1.60	1.24

SUMMARY

1. Seedling carrots contain less alpha and beta carotene than they do carotenols and other strongly adsorbed carotenoids. During the

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[4] The temperature coefficient (Q_{10}) of a process is defined as the number of times that the rate of the process increases with 10° C. rise in temperature.

development of the carrots the concentration of carotenols per unit fresh weight remains fairly constant but there is a steady increase in amounts of alpha and beta carotenes.

2. Lighter portions of carrot roots have a higher absolute value of the more strongly adsorbed pigments (zeta, gamma, and the carotenols) than do the more highly colored portions of the roots. There is a higher proportion of these strongly adsorbed pigments in the xylem. The phloem has a high proportion of beta carotene.

3. Carotene breakdown in air in dehydrated ground carrots stored at 40° C. showed an initial period of no degradation or very slow degradation, a second period of rapid degradation, and a third or final period when pigment breakdown was very slow.

4. The rate of breakdown of alpha and beta carotene was similar to that of the total pigment complex. The more strongly adsorbed pigments (zeta, gamma, and the carotenols) remained fairly constant in concentration, showing a decrease only when the rate of breakdown of alpha and beta decreases.

5. The rate of breakdown in air during the second or rapid period, for any given experiment was constant. In pure oxygen an exponential curve was obtained. Apparently pigment breakdown in ground dried carrots in air is not proportional to pigment concentration but it is related to concentration when the sample is stored in oxygen.

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THE INFLUENCE OF SELECTED ANTIOXIDANTS ON DEHYDRATED CARROTS [1]

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INTRODUCTION

Certain external factors influencing the rate of carotenoid degradation in dehydrated carrots have been considered in a companion paper. This paper reports: (1) The influence of certain internal factors such as carotene and starch content on pigment degradation; and (2) the ability of certain selected antioxidants to retard the rate of pigment degradation.

MATERIAL AND METHODS

The source of carrots and methods of pigment analysis have been reported by Stocking and Weier (*6*).[2]

Antioxidants were generally applied by soaking the blanched dice in solutions of selected chemicals. Except for sodium metabisulphite no effort was made to determine the amount of antioxidant within the dehydrated dice. While this is an important aspect of the problem the major interest was first to determine whether or not the carotene could be protected.

In the majority of the experiments herein reported the SO_2 content was approximately 1,000 parts per million. This concentration of SO_2 in the dehydrated dice was obtained by soaking the blanched dice in a 0.12-percent solution of $Na_2S_2O_5$.

Unless otherwise indicated the samples for the accelerated breakdown tests were ground, after dehydration, to pass a 20-mesh screen and be retained on a 48-mesh screen. They were stored in air at 40° C.

The problem was to determine whether or not the pigment in dehydrated carrots could be protected by antioxidants and if so which antioxidants were of highest value. All experiments were conducted in duplicate, unpromising antioxidants were immediately discarded, others were tested a second time, and the best (such as pyrogallol) were tested five or six times or more under varying conditions. Pyrogallol was tested 10 times over a 2-year period.

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RATE OF PIGMENT BREAK-DOWN IN GROUND SAMPLES UNDER
ACCELERATED STORAGE CONDITIONS

VARIATION IN TOTAL PIGMENT CONTENT

Previous reports on the pigment content of carrot roots indicate that the pigment increases to a maximum at from 16 to 22 weeks after planting; thereafter the pigment remains constant, (1), (3). Our own results confirm these experiments.   The pigment increases rapidly during the active growing season, after which it remains practically constant.   This applies to carrots grown during both summer and winter seasons.   Roots resulting from September sowings did not accumulate so much pigment as those planted in March.   Hansen (*3*) reports similar results with carrots grown in Oregon.

Cessation of pigment deposition, in general, seems to be correlated with a natural decrease in the growth rate.   In Oregon and New York this coincided with the onset of fall weather; in the University of California's garden at Davis, deposition of pigment ceased in early September, with a month of warm weather remaining.   Pigment deposition was halted in the spring by bolting.   The relationships between deposition of pigment and season of the year is shown in figure 1.
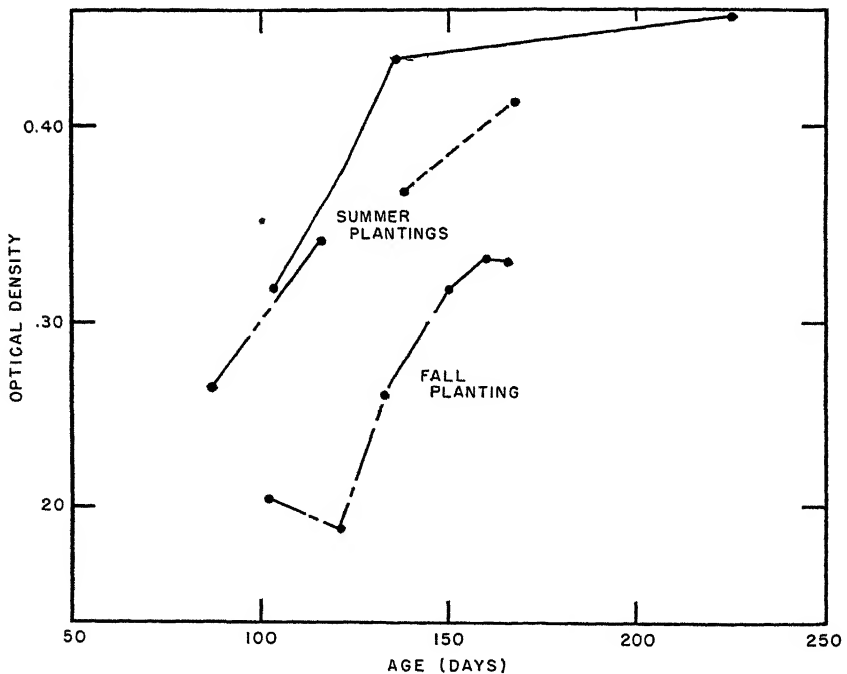

FIGURE 1.—Natural increase in carotenoid content in carrot roots harvested at various ages and at different seasons.   Optical density is based on a sample of one-tenth of a gram of dehydrated carrots to 100 milliliters of solution.

This natural variation in pigment content increases the difficulty of accurately comparing the rates of pigment break-down in dehydrated carrots. The extreme variation, occurring between young and old summer-grown roots may be as much as 200 percent.

During the course of the investigation 24 separate experiments in which the diced carrots received no treatment other than blanching and drying were carried out. These experiments included carrots of all ages and of varying total pigment contents. They cover three growing seasons; the summer of 1945, the fall and spring of 1945–46, and the summer of 1946.

A comparison of the results of these experiments showed that as the initial pigment content increased the percentage of pigment remaining after a 20-day storage period also increased. This means that comparisons between different experiments based on percentages may lead to erroneous conclusions, in that a larger proportion of pigment remaining after a stated time interval may be caused by a larger initial pigment content rather by a variation in treatment. For the same reason a direct comparison between the absolute amount of pigment decomposed or of pigment remaining after a stated period of time may lead to erroneous conclusions.

When the logarithm of the amount of pigment oxidized in 20 days is plotted against the initial pigment concentration a fairly direct relationship is observed. An increase in the initial pigment content results in a proportional increase in the amount of pigment oxidized. The agreement between different experiments is, however, not as close as would be expected under more ideal conditions, such as known concentrations of pure carotene in a pure oil (Bickhoff and Williams *2*).

The results of these 24 experiments are shown in figure 2. The individual points represent the log of pigment oxidized in 20 days (optical density of 100-milliliter acetone extract of 0.100 gram of ground carrots), in a given experiment, plotted against initial pigment content.

A regression line was run for these 24 points. It is the center broken line in figure 2. The significance of this line is at the 2 percent level and the standard error is ±.013. The lined area on either side of the regression line represents this standard error. It may be assumed, therefore, that if the plot of the log of the pigment oxidized in 20 days against the initial pigment content, obtained in any experimental treatment, falls within this lined area the rate of pigment break-down is that which might be expected for a blanch-only experiment. If a particular treatment results in a rate of break-down which when plotted falls above this lined area, this treatment may be considered as accelerating the rate of break-down. If, on the other hand, the results of a given treatment fall below the lined area it may be assumed that that treatment is affording more protection to the pigment than blanching alone.

It must be remembered that there are two aspects to the rate of pigment break-down as measured by the amount of pigment oxidized during 20 days: First, the length of the induction period and, second, the rate of break-down during the straight-line period. In general,
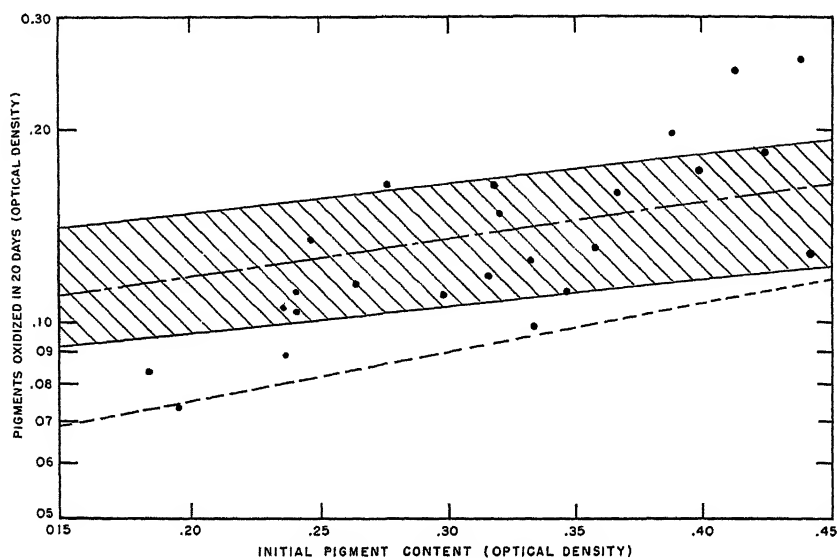
FIGURE 2.—A comparison of amount of initial pigment content (represented by optical density) with amount of pigment oxidized in 20 days (also represented by optical density) in dehydrated ground carrot samples stored in air at a temperature of 40° C. Lined area indicates range of variation in pigment oxidized in 20 days; broken line in center of lined area is the regression line. Results obtained in 24 blanch-only experiments.

it has been found that a lengthened induction period results in a slower rate of break-down during the straight-line period.

From a practical viewpoint any treatment to be of value must protect the pigment to an extent which is greater than the best protection afforded by blanching. Thus in the authors' system of comparison the results of a treatment to be considered good must fall on the graph in figure 2 below the lower evenly broken line, which indicates the upper limit of protection afforded by simple blanching.

THE EFFECT OF STARCH CONTENT ON CAROTENE STABILITY

If diffusion of oxygen into the tissue controls the rate of pigment breakdown under certain conditions, it seems logical that starch gelatinization may protect the tissue from this break down as Reeve (5) has suggested. Mann and Weier (4) pointed out the great variability in the starch content of individual carrots of a single strain. In order to test whether such variation in starch content would materially affect pigment break-down carrots were tested for starch and separated into high and low groups on the basis of a rough quantitative determination with iodine solution.

After their separation into high and low starch, the roots were split and the xylem separated from the phloem so that a series of four types was obtained: High-starch xylem, low-starch xylem, high-starch

phloem, and low-starch phloem. The amount of pigment broken down in 20 days was compared in each case with the normal blanch curve. Although the high-starch xylem and high-starch phloem both showed some less break-down of carotene than that in the low-starch tissue, the results were not sufficiently different to warrant further investigation.

PREPROCESSING STORAGE

Commercial processing procedures are often delayed beyond harvest time, necessitating certain post-harvest storage. A series of experiments was conducted to test the possibility that such storage might affect the rate of subsequent pigment degradation. Carrots were harvested and stored under the conditions indicated in table 1 and were then processed. When the amount of pigment broken down in them in 20 days was compared with the amount of pigment in the normal untreated blanched carrots no significant differences were observed in the rate of carotene break-down.

INFLUENCE OF SELECTED ANTIOXIDANTS

GROUND SAMPLES

As already indicated, Weier (7) and Weier and Stocking (8) have shown that under certain conditions the pigments in blanched and in blanched and dehydrated carrots could be protected to a certain extent by selected antioxidants. In our own laboratory 3-year-old samples of antioxidant-treated carrots still show a brilliant carrot color, whereas the color of comparable blanched-only and blanched—$Na_2S_2O_5$—samples are greatly faded.

TABLE 1.—*Comparison of amount of initial pigment and pigment oxidized in 20 days in carrots grown in the greenhouse and in the field, when stored after being harvested*

Postharvest storage conditions	Initial pigment content	Pigment oxidized in 20 days	Postharvest storage conditions	Initial pigment content	Pigment oxidized in 20 days
	(*Optical density*)	(*Optical density*)		(*Optical density*)	(*Optical density*)
Carrots grown in pots in greenhouse:			Carrots grown in field—Con.		
			32° F., 6 days, topped_____	0.274	0.132
Control _____	0.325	0 117	0° F., 6 days, topped_____	.340	.109
32° F., 5 days_____	.352	.127	Control_____	.311	.123
0° F., 5 days_____	.372	.134	70° F., 4 days, topped_____	.282	.116
Carrots grown in field:			70° F., 4 days, not topped___	.289	.125
Control_____	.330	.099	40° F., 4 days, topped_____	.311	.146
95° F., 6 days, topped _____	.281	.101	40° F., 4 days, not topped___	.320	.154
65° F., 6 days, topped_____	.261	.081			

Previous reports of the action of antioxidants have given only the percentage of pigment remaining in the stored sample after relatively short periods of storage. In table 2 all experiments are compared in which antioxidants were applied to blanched diced carrots which were then dehydrated, ground, and stored. Initial pigment content, the
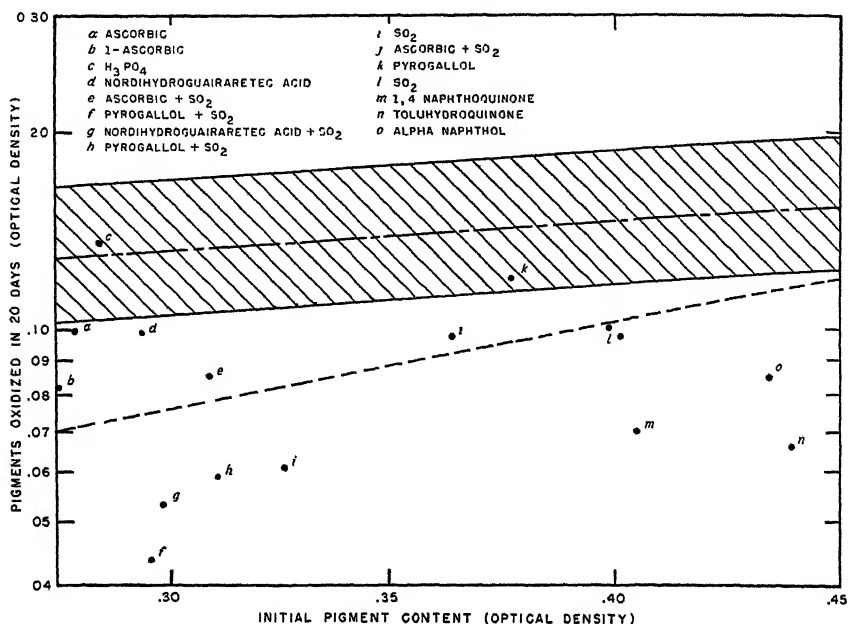
FIGURE 3.—Results of selected antioxidant experiments plotted on the blanch-only treatment curve of figure 2. Points above lined area indicate pigment degradation at a rate faster than blanch only. Points below lined area indicate a retardation in rate of breakdown.

length of induction period, and the pigment oxidized in 20 days are considered. In figure 3 the results of selected runs are plotted on the standard blanch curve shown in figure 2. Attention is called to the following points. The results of the SO_2 treatments fall below the best blanch line, as is to be expected, but in two out of the three cases only slightly so. It seems likely that a statistical analysis of a large number of sulfite treatments would probably indicate a variation of results similar to that observed for blanch only. Such antioxidants as nordihydroguaiaretic acid, ascorbic acid, and pyrogallol when used alone did not afford increased protection of the pigments. When, however, $Na_2S_2O_5$ was added to the pyrogallol so as to make a solution of 0.12 percent $Na_2S_2O_5$ and 0.3 percent pyrogallol in which the blanched carrots were soaked for 5 minutes, the degradation of the carotene pigments was greatly retarded. Toluhydroquinone and 1,4 naphthoquinone, without the addition of $Na_2S_2O_5$ were effective in reducing the rate of pigment break-down.

The pyrogallol used in these experiments was a sample of Merck photographic pyrogallol that had been in the laboratory for at least 15 years. It was slightly tan, indicating a small amount of oxidation. Chemically pure pyrogallol was substituted for the old sample in the late spring of 1947 and, surprisingly, afforded little or no protection

TABLE 2.—*Results of antioxidant treatment of ground, dehydrated carrots*

Treatment after blanch	Initial pigment content	Pigment oxidized in 20 days	Induction period	Days to 80 percent	Relation to blanch
	Optical density	*Optical density*	*Days*		
Soaked in distilled water for 5 minutes	0.42	0.251	3	9	Poor.
	.528	.285	4	10	Do.
	.290	.147	0	8	Do.
	.397	.139	2	12	Do.
Citric acid	.301	.141	0	9	Do.
Tartaric acid	.287	.140	1	9	Do.
Phosphoric acid	.284	.127	0	9	Same.
Ascorbic acid	.280	.101	4	13	Do.
1-ascorbic acid	.275	.082	4	15	Better.
1-ascorbic acid + Na₂S₂O₅:					
Before blanch	.305	.094	4	14	Do.
Post blanch	.345	.120	2	12	Same.
Partially dry	.365	.109	5	15	Better.
Before blanch + vacuum	.364	.098	5	16	Do.
Ascorbic acid	.312	.087	0	9	Do.
Ascorbic acid + Na₂S₂O₅	.318	.085	0	15	Do.
Pyrogallic acid	.374	.113	0	7	Same.
Pyro + Na₂S₂O₅	.311	.059	3	21	Better.
Pyro + Na₂S₂O₅:					
Preblanch	.270	.059	5	17	Do.
Post blanch	.296	.044	10	23	Do.
Partial dry	.291	.037	9	25	Do.
Na₂S₂O₅	.401	.098			Do.
	.326	.062			Do.
	.396	.103			Do.
N. D. G. A.[1]	.291	.100	0	10	Same.
N. D. G. A. + Na₂S₂O₅ [1]	.298	.053	8	22	Better.
Hydroquinone	.328	.117	0	7	Same.
Hydroquinone + Na₂S₂O₅	.316	.052	5	21	Better.
Toluhydroquinone	.443	.099	7	19	Do.
Toluhydroquinone	.440	.066	12	18	Do.
Hydroquinone triacetate	.443	.099	8	19	Do.
1, 2 Naphthoquinone	.423	.095	14	24	Do.
2-4 Methyl-1-4 naphthoquinone	.389	.096	6	14	Do.
b Naphthol	.435	.083	10	20	Do.
1,4 Naphthoquinone	.412	.069	11	21	Do.
1,5 Dihydroxy-naphthalene	.404	.092	5	14	Do.
40 percent Acetone leach	.418	.201	5	11	Poor.

[1] Nordihydroguaiaretic acid.

to the carrot roots being tested. These roots were large and contained an unusually high concentration of carotene pigments. The series of experiments on these high carotene roots, in which blanch, leach, old and new c. p. pyrogallol are compared, is shown in figure 4.

The protection afforded by blanching-alone was somewhat poorer than the average as is shown by the plotting of these points. It is possible that in roots with this large absolute concentration of pigments the rate of break-down after blanching may be higher than the curve of figure 2 would indicate. This series of experiments again indicated that the old sample Merck photographic pyrogallol considerably retarded the rate of pigment degradation. In all, 10 separate experiments, extending over a 2-year period, were run, using this old photographic pyrogallol. In every case considerable protection was afforded the carotene pigments. The authors were unable to discover the reason for the difference in effectiveness of the old and the new c. p. samples of pyrogallol. The possibility was considered that impurities, such as the tannins, might be the factor supplying the protection. Tannic acid (Baker) was tested with negative results.

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Under the conditions of our experiments, selected antioxidants, both alone and in conjunction with $Na_2S_2O_5$ afford greater protection than blanch-only and blanch-sulphite treatments to the carotene pigments in dehydrated, ground carrots stored at $40°$ C.
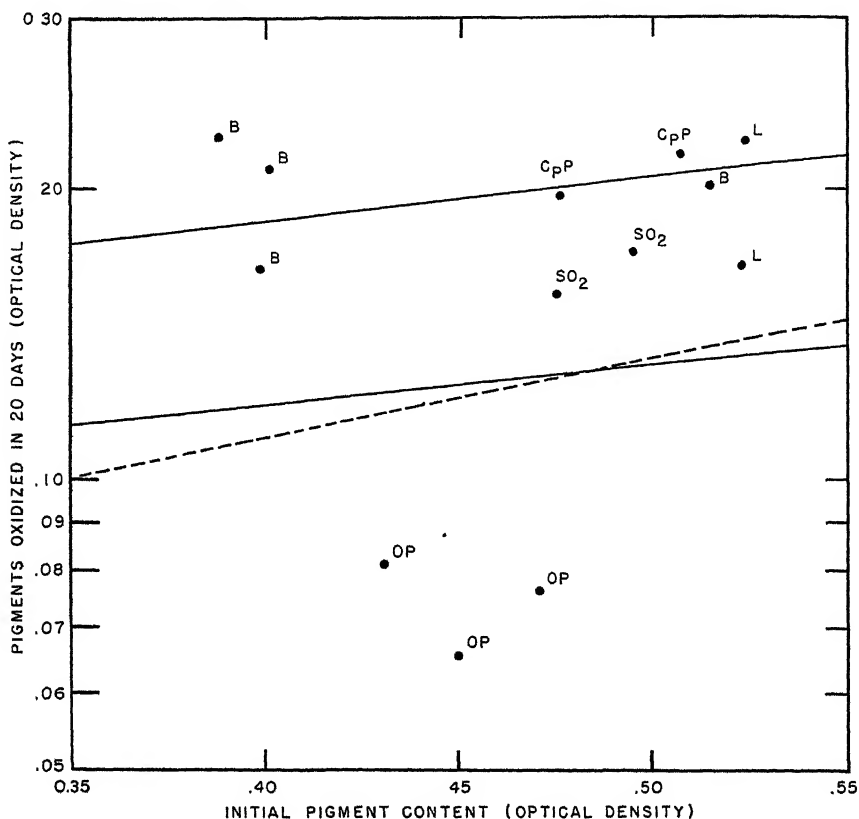
FIGURE 4.—A comparison of blanch-only treatment, (B); $Na_2S_2O_5$, (SO_2), leach (L); c. p. pyrogallol, (CpP), and old Merck photographic pyrogallol, (OP). Points plotted on blanch-only curve. Lower points indicate retardation of pigment break-down.

A study of the degradation of individual pigment components was undertaken to determine whether one antioxidant is more effective than another in protecting any of the pigments. Figure 5 shows the effect of treatment with 0.3 percent $Na_2S_2O_5$ on the break-down of the separate fractions. Here again the alpha and beta carotenes show the same shape curves with the increased induction period characteristic of the sulfite-treated carrots. The treatment apparently affected break-down of the individual components to about the same degree. Similar results were obtained with other antioxidants such as pyrogallol and ascorbic acid.

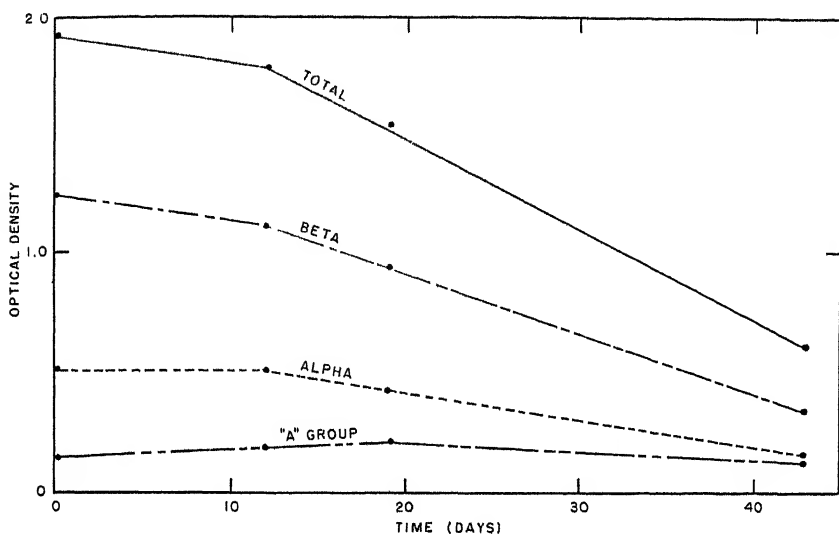FIGURE 5.—Rate of break-down of total complex and individual pigments after treatment with 0.12 percent $Na_2S_2O_5$. Ground dehydrated carrots stored at 40° C. optical density based on pigment from 1 gram of dehydrated carrots in 200 ml. of solution. Group "A" refers to all carotenoid pigments more strongly adsorbed than beta carotene.

DICED SAMPLES

In testing the effectiveness of antioxidants in protecting the carotenes in dehydrated diced carrots, the only change in procedure from that using ground samples was to store the material as dice for a 3-month period. In conjunction with all experiments on dice, separate 5-gram samples were ground and stored, as in other accelerated tests. Thus, material from a single given processing was stored both in diced and in a ground state. Treatments given the diced carrots are as follows: $Na_2S_2O_5$; $Na_2S_2O_5$+gelatin; $Na_2S_2O_5$+c. p. pyrogallol; $Na_2S_2O_5$+old Merck pyrogallol; c. p. pyrogallol; old Merck pyrogallol; 2-4 naphthoquinone; toluhydroquinone; Na_2SO_3+c. p. pyrogallol; $Na_2S_2O_5$+nordihydroguaiaretic acid; $Na_2S_2O_5$+gallic acid; gallic acid.

In no treatment was the rate of pigment break-down in the dice significantly different from that in the control blanch-only. This was true even after soaking in the 0.03 percent old Merck pyrogallol: 0.2 percent $Na_2S_2O_5$ solution. In the ground aliquot from this same sample of dice the rate of pigment break-down was retarded as usual. The old pyrogallol then is able to protect the pigment in the ground dehydrated carrots but not in the diced carrots.

Figure 6 shows the rates of pigment degradation for blanch-only treatment, $Na_2S_2O_5$, old pyrogallol, and old pyrogallol plus $Na_2S_2O_5$. The upper curve with the low slope represents the rate of pigment break-down in the dice. The differences between these four upper curves are not significant. The left-hand curves in each small graph represent the pigment break-down in the ground material that was
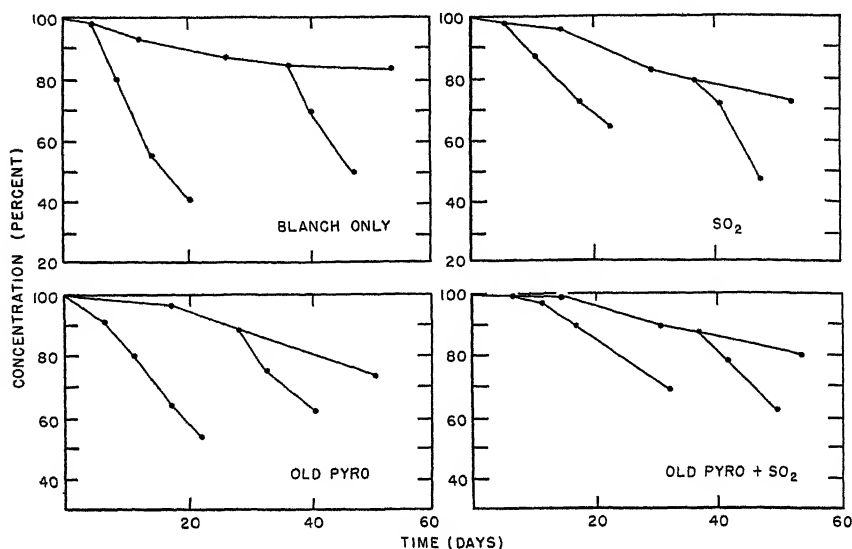
FIGURE 6.—Comparison of rate of pigment break-down in four samples of carrot dice treated as indicated. Upper curves dice stored at 40° C. for 3 months. Left-hand curves represent ground samples stored immediately after preparation. Right-hand curves represent ground samples prepared from dice after 30–40 days' storage.

stored as soon as prepared. Note that the old pyrogallol plus $Na_2S_2O_5$ gives considerable protection. The right-hand curve of each small graph represents the rate of break-down in a sample taken from the diced material after a month's storage. This sample was ground and stored as in all other accelerated tests. Note that in each case these curves are approximately parallel with the curves showing pigment break-down in the ground samples stored immediately after preparation. It seems evident that the rate of break-down and the effectiveness of the antioxidant are greatly influenced by the size of the pieces of stored dehydrated carrots (1).

EFFECT OF PARTICLE SIZE ON PIGMENT STABILITY

While most of the experiments were carried out on ground material and some were carried out on dice, one set of experiments was run on a graded series of particle sizes to determine the effect of particle size on pigment stability. Carrots for this series were prepared, blanched, and divided into two groups. One group was dehydrated without further treatment and the second group was treated with 0.3 percent Merck's pyrogallol prior to dehydration. After drying, the carrots were ground in a coarse food grinder and placed on a series of standard sieves on a Rotap machine. The two sets were thus separated into four particle sizes 28 to 48 mesh, 14 to 28 mesh, 8 to 14 mesh, and greater than eight mesh. The rate of pigment break-down for these eight samples was determined and is shown in figure 7.
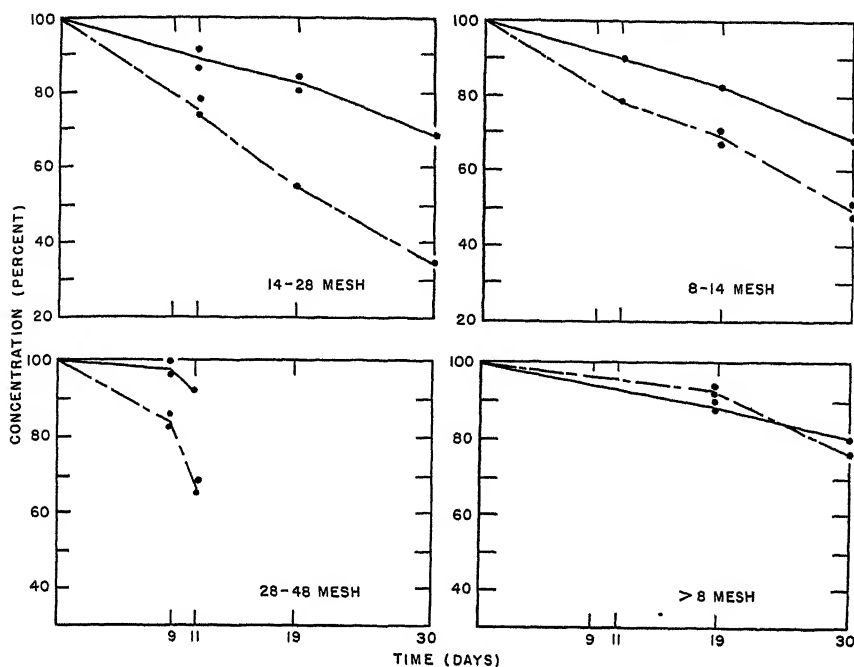
FIGURE 7.—Influence of size of dehydrated carrot particle on rate of pigment break-down.   Solid line, in all cases, represents old pyrogallol treatment and broken line, blanch-only treatment.

It is apparent from these curves that the rate of carotene degradation is a function of particle size and that smaller particle have a very rapid break-down.   The protective action of pyrogallol (solid line) on this break-down is apparent when the particles are less than 8-mesh in size.   However, if the particle size is increased sufficiently there is a decrease in the rate of pigment loss from the untreated pieces but little if any change in the slower rate of break-down of the treated pieces.   Apparently the protective action of pyrogallol can be duplicated by increasing particle size but with change in size there is little or no change in effective protection when an antioxidant such as pyrogallol is used.[3]

PREHARVEST SPRAY TREATMENT

A series of preharvest spray treatments of young carrots was undertaken to test the possibility of stabilizing the carotene in the harvested dehydrated material either by changing the phospholipid content or

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[3] When the samples for these experiments on diced carrots were prepared, aliquots for $Na_2S_2O_5$ and $Na_2S_2O_5$+old pyrogallol were stored in a basement with a temperature range of 16° to 20° C.   These samples have been in storage about 30 months.   No analysis have as yet been conducted, but the color of the $Na_2S_2O_5$—only sample is considerably paler than that of the $Na_2S_2O_5$+old pyrogallol. Complete results of this storage test will be reported at a later date.

by incorporating antioxidants into the carrots. Phospholipids are more stable than free fatty acids and while the probability that changes in carrot phospholipids could be affected by spray treatment was low, it seemed that positive results, if obtained, would be of such significance as to make the experiment worth while. The materials used, time of treatment, and results are shown in table 3. The results when compared with the standard control curve figure 1 show that none of these treatments increased the stability of the carotene in the processed carrots sufficiently to be of practical importance although the June 8 choline chloride and the July 27–Aug. 10 kerosene treatments caused significantly less breakdown than the untreated blanches and indicate the desirability of future work in preharvest spray treatment.

TABLE 3.—*Effect of preharvest spray treatments on the breakdown of pigment in carrots*

Spray	Initial pigment content	Pigment oxidized in 20 days	Spray	Initial pigment content	Pigment oxidized in 20 days
	Optical density	*Optical density*		*Optical density*	*Optical density*
Series 1:[1]			Series 2—Continued		
Na2H PO4	0.363	0.145	Glycerine	0.243	0.092
Glycerine	.344	.148	Choline chloride	.282	.121
Choline chloride	.314	.139	Ethanolamine	.231	.089
Ethanolamine	.333	.157	Na2S2O5	.246	.103
Na2S2O5	.311	.128	Na–glycerophosphate	.215	.095
Na–glycerophosphate	.267	.116	Dreft	.278	.117
Dreft	.316	.117	Series 3:[3]		
No treatment	.315	.118	Kerosene	.183	095
Kerosene	.214	.070	Kerosene+N. D. G. A	.174	.082
Kerosene+tocopherols	.330	.135	Kerosene+tocopherols	.222	.080
Stove oil	.250	.095	Stove oil	.222	.087
Stove oil+tocopherols	.234	.089	Stove oil+N. D. G. A	.233	.100
Series 2:[2]			Dreft	.262	.113
H3PO4	.239	.091	Ethanol amine	.187	.095
Na2H PO4	.252	.098	No treatment	.184	.083
NaH2PO4	.265	.101	Choline chloride	.165	.061

[1] Sprayed July 27 and Aug. 10; 84, and 98 days after sowing.
[2] Sprayed July 27, 84 days after sowing.
[3] Sprayed June 8; 35 days after sowing.

SUMMARY

Carotene content varied in different samples of carrots over a 2-year period by 200 percent or more. The initial pigment content does play a parte in rate of carotene degradation. For this reason the ratio of the logarithm of pigment oxidized over a stated period of time to initial pigment content provides the best comparison of rates of break-down between experiments widely separated in point of time.

The pigment in blanched, dehydrated, and ground carrots stored at 40° C. is protected by the following antioxidants to a much greater extent than by blanching only or by blanching plus $Na_2S_2O_5$: pyrogallol (old Merck photographic) plus $Na_2S_2O_5$; nordihydroguaiaretic acid + $Na_2S_2O_5$; toluhydroquinone; and 1,4 naphthoquinone.

No selective action of any antioxidant on any individual carotene component was apparent. In all cases the rate of break-down of alpha and beta carotenes follows that of total pigment complex (crude acetone extract), while concentration of the more strongly adsorbed pigments remains fairly constant.

The rate of pigment break-down in dice is not retarded by the impregnation with antioxidants. Size of dice is the determining factor in rate of pigment break-down and of the effectiveness of certain antioxidants.

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DEVELOPMENT OF PIGMENT IN THE FUR FIBERS OF AGOUTI-COLORED RABBITS [1]

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INTRODUCTION

Fur is made up of two types of fibers: Guard hairs, of which there may be more than one kind, and underfur (*19*).[3] The guard hairs have a greater diameter than the underfur and project beyond the dense covering of the underfur fibers.   Ideal pelts possess clear color and have "life."   They are silky and full-furred, have sufficient guard-hair coverage, and the skins are of good size.   Color plays a very large part in market values since it contributes the key quality to the beauty of furs.

Varying intensities of melanin, the principal pigment found in all animal hair, impart to the fibers colors ranging from black through many shades of gray, and from brown and tans to yellows.   Sometimes animal hairs appear to be blue, but on closer examination this color proves to be a form of slate gray.   "Tyndall blue" is the term used by Fox (*13*) to describe the blue shade derived from melanin.

Skins that show a pleasing contrast in color between underfur and guard hair are preferred to skins that do not show contrast.   In mink farming, one of the objectives is a rich dark-brown over-all color; the clear dark-brown guard hairs contrast with the underfur, which appears to be blue.   In various light phases of genetic mutations in mink and fox, the underfur is pale gray or sky blue; the guard hairs are slightly darker.   In the silver fox, the black tip of the guard hairs together with the pure white band, located just below this tip, produces a veiling effect; this combination enhances the color contrast. The underfur is gray or blue gray.

Although scattered and fragmentary material is available on pigment studies in the mouse (*12, 22, 30, 31*), guinea pig (*7, 32, 35*), cow (*7*), and sheep (*8*), practically no work of this nature has been reported on animals whose skins are used commercially as furs.   In

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August 1944, research on pigment development of the agouti-colored rabbit (*Oryctolagus cuniculus*) [4] was initiated in the Fish and Wildlife Service, United States Department of the Interior, to be carried out in its fur fiber laboratory at Beltsville, Md.[5] In this animal, as in a number of others, the laying down of pigment in alternate dark and light bands produces a mottled pattern. The guard hairs of this rabbit are black at the tip, followed by bands of orange, dark brown or black, and light gray. The underfur is gray for the greater part of its length and is banded at the distal end in most of the fibers.

The agouti-colored rabbit was selected as the animal for study in this investigation for several reasons. Since the guard hair is tipped with black, there is a heavy concentration of melanin in the early stages, which facilitates the study of pigment development in the fur fiber. Also, the gestation period in the rabbit is short, approximately 31 days, and embryos can be obtained within relatively short periods of time. This animal is readily available, is docile in temperament, and can be handled without much difficulty.

MATERIAL AND METHODS

To study the sequence of pigment development in the agouti-colored rabbit, blocks of tissue were removed from two fetuses taken at 18 and 25 days; from nine juvenile rabbits at 1 to 5 days and at 8, 10, 12, and 25 days after birth; and from a live adult animal;[6] age unknown.

Bouin's, 5-percent formalin, and Zenker's (*9*) were used as fixatives. Bouin's, according to the method outlined by Becker and Roudabush (*1*), gave best results. Tissue blocks were cut at an angle to the skin surface and oriented to obtain longitudinal sections of the hair follicles. It was not always possible to get exact orientation, with the result that hairs sometimes appeared in cross or tangential section. If freshly cut paraffin sections are placed on a slide and the paraffin removed by applying a few drops of xylol, the presence of pigment in the hairs can be used as a guide in determining the proper orientation.

Various stains were used in this investigation. Haematoxylin and eosin show up the structures of the skin, but haematoxylin masks the pigment to some extent. Cell outlines are also well defined with iron haematoxylin and orange G. Silver nitrate was used when it was desired to intensify the appearance of pigment granules, and the slides were sometimes counterstained with pyronin methyl green (*23*). The orcein and Giemsa stain described by Pinkus (*23*) proved to be valuable in the definition of the inner root sheath. Mallory's triple connective tissue stain (*17*) and methyl green were also used.

As a mounting medium, gum dammar was superior to Canada balsam since it dried faster and seemed not to yellow with age.

The dopa (shortened term for dihydroxyphenylalanine) reaction as worked out by Bloch (*5*) was used to determine the potentialities of

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[4] The agouti-colored rabbits were provided through the courtesy of Dr. Don R. Coburn, Patuxent Research Refuge, Bowie, Md.

[5] All the activities pertaining to the production of fur animals in captivity (fur farming), including domestic rabbits, were transferred to the U. S. Department of Agriculture June 30, 1946, by act of Congress.

[6] Blocks of skin from the adult rabbit were excised by Dr. Louis C. Heemstra of the Bureau of Animal Industry.

pigment development, requiring the use of fresh tissue or tissue fixed for a short time in 5-percent formalin. Blocks of skin 3 to 5 mm. thick were fixed in formalin from 2 to 3 hours. Sections ranging in thickness from 15 to 30μ were then cut on the freezing microtome (*14*), rinsed in distilled water, and placed in the dopa solution (*25*). Approximately 20 cc. of the solution was used for 50 sections and kept at 40° C. for 3 hours, with a fresh change of dopa solution after the first half hour and then again after another hour (*32*).

The paraffin section method for the dopa reaction, developed by Becker, Praver, and Thatcher (*3*), revealed more nonspecific blackening around the outer edges of the tissue. Blocks of skin were first treated with the dopa solution at 37° C. for 15 hours, with a change of solution after the first half hour. Following this treatment, the blocks were fixed in Bouin's, carried through the alcohols and xylol, and embedded in paraffin.

PIGMENTATION IN THE HAIR

18-DAY-OLD FETUS

No pigment is evident in the 18-day-old fetus. At this stage, the development of hair is more advanced in the ear (fig. 1) than in other regions of the body. Knoblike aggregations of cells, the hair anlagen, are present in the epidermis, and these cells as they grow downward into the dermis become enclosed in a pocket, the hair follicle. In some cases it appears as though the proliferation of cells of the hair proper gets under way before the definitive papilla takes shape (*28*).
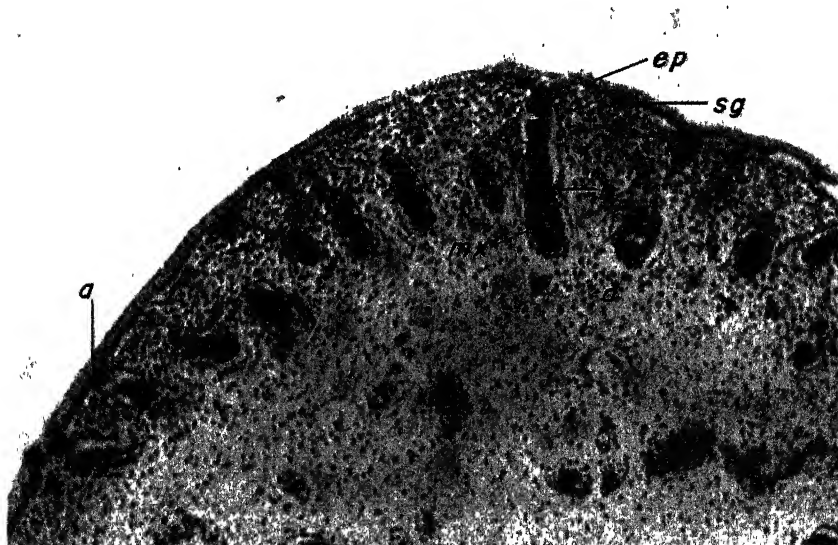

FIGURE 1.—Anlagen and early hairs in ear of 18-day-old fetus. The matrix has taken up the stain (haematoxylin and eosin) more deeply than the surrounding cells: *a*, Hair anlage; *ep*, epidermis; *sg*, columnar cells of stratum germinativum; *h*, downgrowth of hair into dermis; *mx*, matrix; *d*, dermis. × 100.

The papilla is produced by mesenchymal cells and fits into the broadened base of the hair. The mesenchymal cells surrounding the hair root become the connective tissue sheath; this sheath is found only in the guard hairs and usually serves for the insertion of smooth muscle fibers.

The matrix cells of the hair are located in the bulb, just above the papilla. These are the active cells that later give rise to the hair, the inner root sheath, and the pigment cells themselves. The hair and its sheaths—the inner root sheath and the outer root sheath (fig. 3)—are epidermal in character, whereas the connective tissue sheath (fig. 4) and the papilla (figs. 6 and 8) are dermal in origin.

25-DAY-OLD FETUS

Generally, the first pigment cells are seen in the matrix region of the hair in the 25-day-old fetus, but occasionally some may be found in the undifferentiated medulla. These cells are also called melanoblasts and differ from the other epithelial cells in the hair only in that they may carry pigment. Melanin granules in some cases are found along the periphery of the cell (fig. 2), but usually they fill the entire cell (fig. 3). In the study of wool from colored and from white sheep, Hardy and Plitt (*18*) found that the smallest visible units of structure were granules or "particles." These particles were colorless in the white, Lincoln breed of sheep, whereas in the colored, Karakul sheep, they were both colorless and pigmented, the latter being identical with the familiar pigment granules.

At the 25-day fetal stage, the columnar cell make-up of the outer root sheath is clearly recognizable. In figure 4 the outer root sheath
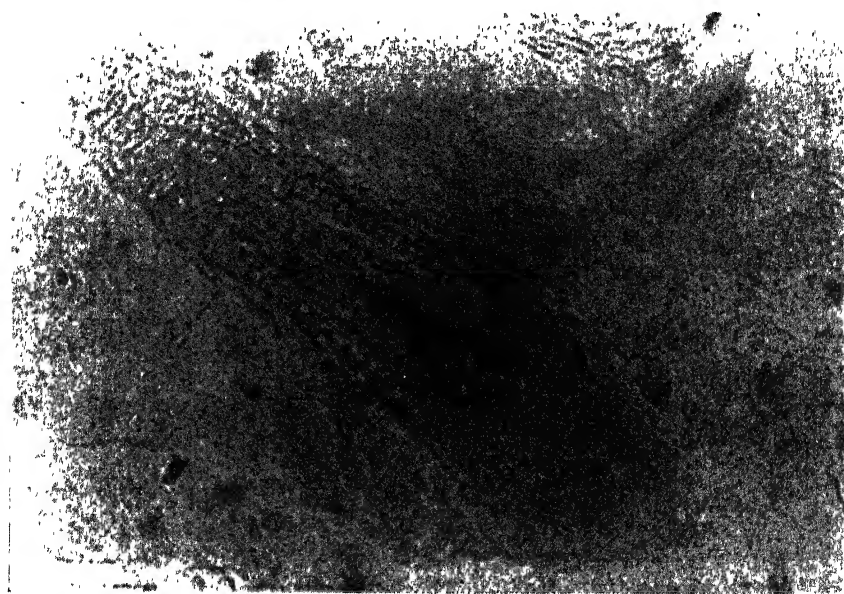
FIGURE 2.—Skin section from 25-day-old fetus; taken from the head and stained with orange G. The pigment granules are arranged along the periphery of the melanoblast, indicated by the arrow. × 400.

FIGURE 3.—Skin section from 25-day-old fetus; taken from the head and stained with orange G: *or*, Outer root sheath; *ir*, inner root sheath; *mel*, fully pigmented melanoblast; *d*, dermis.   × 400.

FIGURE 4.—Early development of hair in skin from head of 25-day-old fetus; stained with haematoxylin and eosin: *sg*, Basal cell layer of epidermis (stratum germinativum); *irt*, tip of inner root sheath; *or*, outer root sheath; *mm*, melanoblast in matrix; *cts*, connective tissue sheath; *d*, dermis.   × 200.

can be traced to the basal layer of the stratum germinativum of the epidermis and is seen to be continuous with it.

JUVENILES (1 TO 25 DAYS OF AGE)

In newborn rabbits of the pigmented breeds the color contained in the hairs that lie just below the skin surface is visible through the skin. Rabbits are born naked; however, a sparse covering of hairs that have pierced the skin can be observed with a hand lens. In figure 5 hairs can be seen emerging from some of the follicles of a 1-day-old rabbit. The young are covered with a good hair growth at about 5 days of age.

In the agouti-colored rabbit, histological sections from the juvenile, as well as from the adult, show that the melanoblasts are always rounded in appearance (fig. 6). Branched pigment cells, however, have been reported in the gray rabbit (*26*) and in other animals (*6, 20, 27, 34*).

The cells in the central portion of the matrix pile up in rouleaux to form the medulla of the hair (figs. 7 and 8). In surface view these cells are round, whereas in end view they appear flat and produce a banded effect. In the rabbit the pigment in the medullary cells is granular in structure; both granular and diffuse pigments are found in the cortex (fig. 9). Although granular and diffuse pigments are present in the hairs of most fur-bearing animals, only one form of pigment has been reported for members of some species (*15, 31*), and this may be either diffuse or granular.
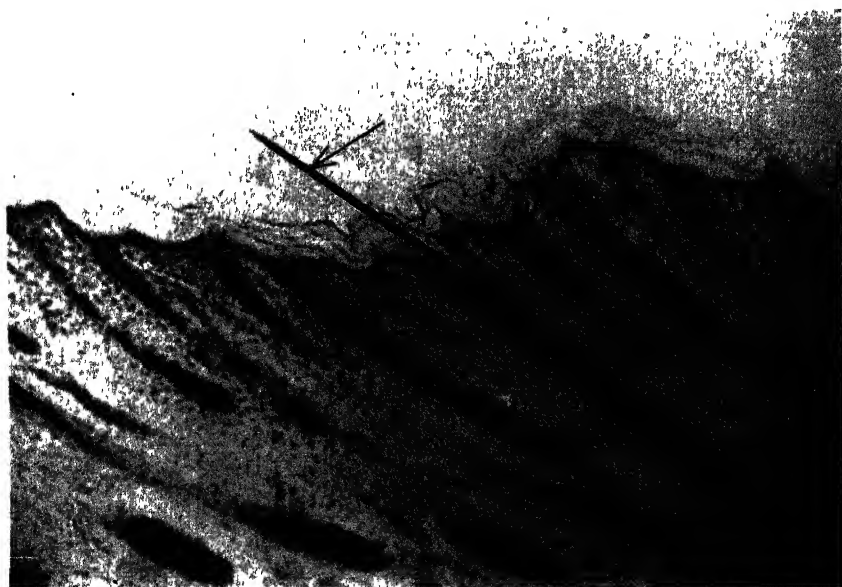

FIGURE 5.—Hair follicles in skin section from loin of a 1-day-old rabbit; stained with haematoxylin and eosin. Arrow points to hair emerging from follicle. X 100.
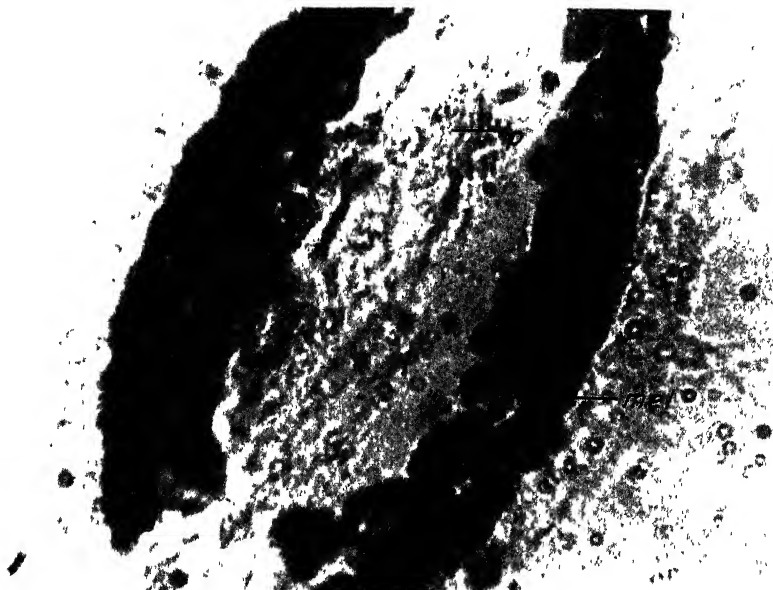
FIGURE 6.—Root end of a hair in the unstained skin from loin of a 2-day-old rabbit. Pigmented cells in the bulb are rounded: *p*, Papilla; *mel*, melanoblast. × 900.

The aggregation of melanin granules (fig. 10), often spoken of as a cap, that appears above the nucleus in the medullae in many animal hairs has led some authors to suggest that melanin is nuclear in origin (*8*). Just what role the nucleus or the cytoplasm plays in melanin elaboration is still to be determined. Conklin (*10*) believes that all cellular differentiations may be formed by the reconstruction of substances that pass through the nuclear membrane and that these substances then enter the cytoplasm in solution.

Boyd (*8*) speaks of single and double nuclear caps of pigment granules in sheep. In some hairs of the agouti-colored rabbit the cap of melanin granules is found at the top and apparently at the bottom of the nucleus (fig. 10).

In skin sections of some of the early stages, pigmented cells were observed to align themselves in a way that suggested polarity. It may be that this cellular polarity (*10*) influences the orientation of pigment granules and that this polarization is responsible, in part, for the so-called capped nucleus. In figure 11 the pigment granules are concentrated at the distal end of the pigment cells. As the hair approaches the skin level, there is a shrinkage of cytoplasm, and air spaces make their appearance between the medullary cells (*34*).

The laying down of dark-colored granules followed by the deposition of light-colored granules was observed in some of the fibers. The difference in intensity (*8, 11, 13, 35*) is illustrated in figure 12; microscopically the light-colored melanin granules appear yellow or light brown.

FIGURE 7.—Pigment cells in the form of rouleaux in the medulla of hair of a 2-day-old rabbit. Skin section was taken from the loin and stained with methyl green: *m*, Medulla; *pce*, pigment cell in end view; *pcs*, pigment cell in surface view. × 100.

ADULT

The replacement of hairs in the adult rabbit is different from the development of fetal hairs in that new anlagen no longer appear in the basal layer of the epidermis and grow downward into the dermis. Hairs are now differentiated from epithelial cells already established in the dermis. That new hairs arise from the already existing follicles has been reported by several authors (*33*, *34*). It is believed that the new hair germ is formed from material of the outer root sheath. This sheath in the fetal stages is seen to be continuous with the stratum germinativum. The part the papilla plays in hair regeneration has been disputed. Hertwig (*21*) is of the opinion that a new papilla is formed, whereas Trotter (*34*) believes that the new hair is nourished by the enlarging old papilla or by a newly formed papilla.

FIGURE 8.—Guard hair in skin from dorsum of a 10-day-old rabbit.   Cells proliferated from the matrix form the medullary columns.   Mallory's triple connective tissue stain was used: *c*, Cortex; *m*, medulla; *p*, papilla, *mx*, matrix; *f*, fat cells.   × 200.
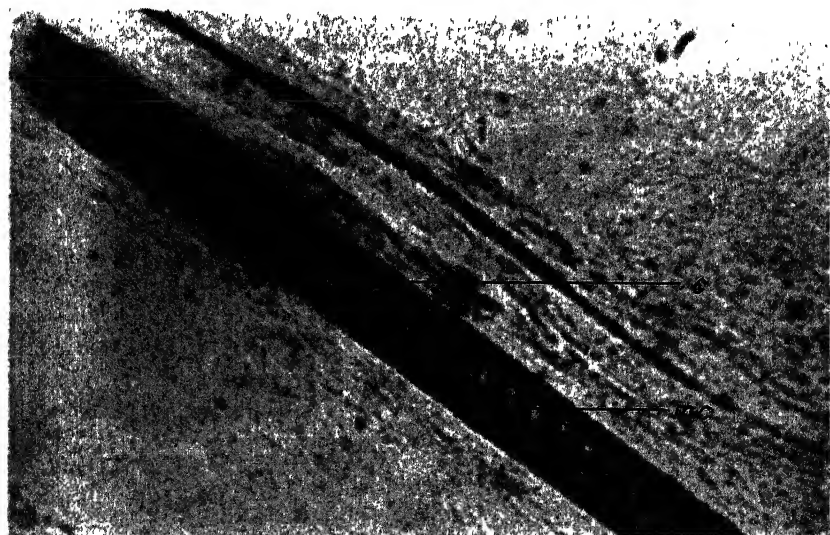
FIGURE 9.—Diffuse and granular pigment in cortex of hair from 2-day-old rabbit.   Skin section was taken from the region of the loin and stained with methyl green: *c*, Cortex; *mc*, outline of medullary cells seen through the cortex.   × 200.
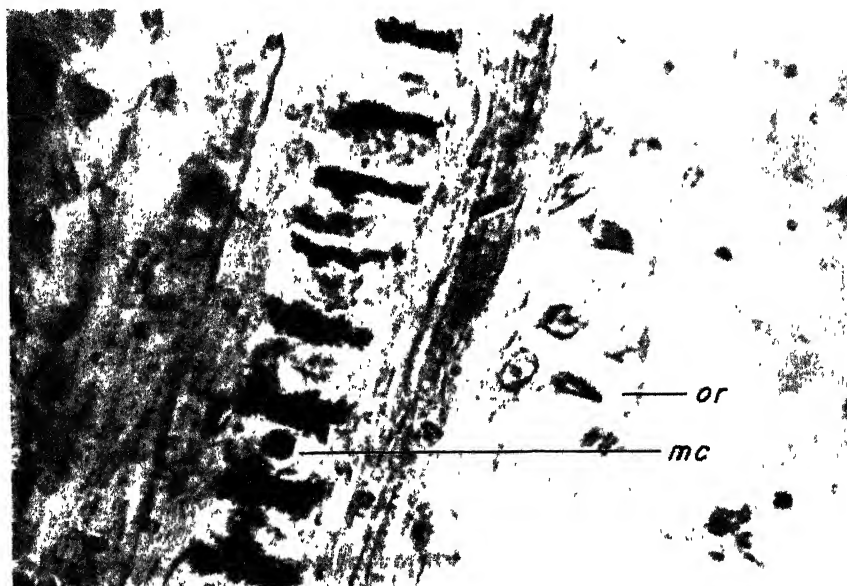
FIGURE 10.—Hair in the skin of 10-day-old rabbit, taken from the dorsum, showing nuclei in the medullary cells. These cells later keratinize and the nuclei disappear. Stained with haematoxylin and eosin: *or*, Outer root sheath; *mc*, medullary cell with caps of melanin granules above and apparently below the nucleus. × 900.
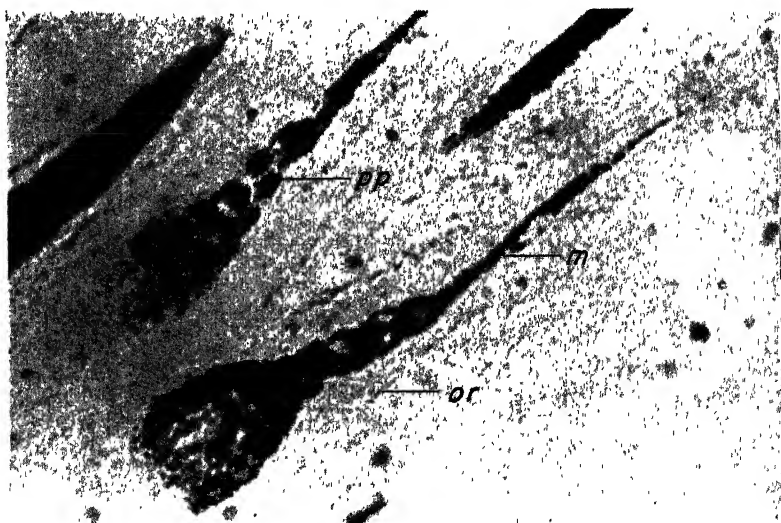
FIGURE 11.—Longitudinal section of unstained skin from 2-day-old rabbit ear showing polarity of pigment granules: *pp*, Pigment cells exhibiting polarity; *m*, medulla; *or*, outer root sheath. × 400.
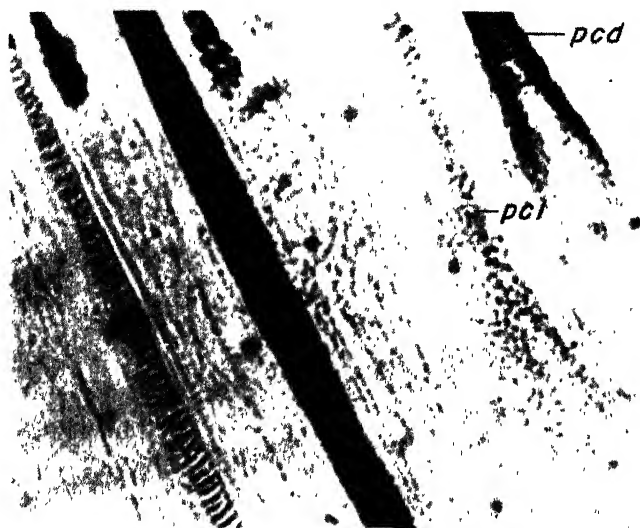
FIGURE 12.—Light- and dark-colored melanin in 2-day-old rabbit hair (unstained), taken from the dorsum: *pcd*, Dark-colored pigment cells; *pcl*, light-colored pigment cells.　× 200.

In the young juvenile the hair covering is fairly uniform, apparently consisting of only one type of fiber.　These fibers are softer in texture than those found in the adult.　In the coat of the older juvenile and in the adult there is a distinction in the hair types (*19*).　The guard hairs are the longer hairs, thicker than the underfur (fig. 13, *A*), and contain two or more columns of medullary cells (fig. 13, *B*).　The underfur fibers are more numerous and contain but one medullary column.

When the hair above the skin measures approximately 3 cm. in length, the papilla diminishes in size, and the hair enters a resting phase.　If the skin of a rabbit whose pelt is prime is examined by blowing into the fur, or by parting the fur, the skin is observed to be creamy white and devoid of pigment spots since the root ends of the hairs have completed their growth cycle and are free of pigment. This period of inactivity is followed by one of renewed differentiation in the hair follicles, and pigment spots (fig. 14) can be seen through the skin (*16*), an indication that pigment is being deposited in the new incoming hairs.　The new hairs, after a period of growth, pierce the skin and for a time lie side by side with the club, or mature, hairs. The new short hairs can be observed macroscopically by running the hand along the body of the animal in a direction opposite to the natural hair flow.

The club hairs that are being shed are recognized by the frayed appearance at the root end (fig. 13, *A*).　Pinkus (*28*) states that the hair forms a brushlike or clublike structure because it is no longer compressed by Henle's layer at its lower end.　Huxley's and Henle's layers, subdivisions of the inner root sheath, usually remain attached to the shedding hair.
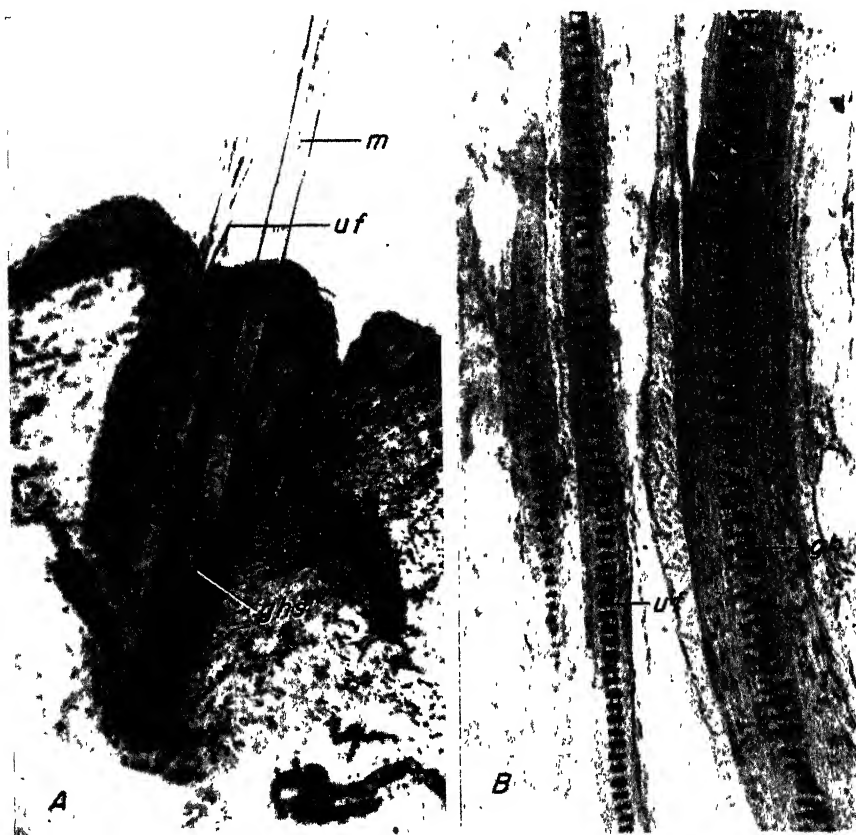
FIGURE 13.—*A*, Guard hair and underfur fibers from the loin region of an adult rabbit. The guard hair shown is in the process of being shed; attention is called to the brushlike structure at the root end. Stained with haematoxylin and eosin. × 100. *B*, Guard hair and underfur fibers from dorsum of 10-day-old rabbit. Stained with Mallory's triple connective tissue stain. × 200. *m*, Base of the medulla in guard hair; *ghs*, brushlike structure of shedding hair; *gh*, guard hair; *uf*, underfur.

PIGMENT IN·THE SKIN

In untreated slides, i. e., slides which are not tested for the dopa reaction, pigment in the skin was observed only in the basal layer of the epidermis in the rabbit ear. The two layers of the epidermis in figure 15 can be easily distinguished. The outermost layer, the stratum corneum, is the most highly keratinized and the cells nearest the surface are constantly being sloughed off. The basal cells of the stratum germinativum, the innermost layer, supply the rest of the epidermis with new cells. Pigment was found nowhere else in the epidermis and, except for the sections treated for the dopa reaction (figs. 16 and 17), was never observed in the dermis.

Both dermal and epidermal pigmented cells have been reported for man, monkeys, some guinea pigs, the gray rabbit, and the gray house

FIGURE 14.—Right side of adult agouti-colored rabbit showing newly developed pigment spots. The hair of this side was removed by means of an electric clipper.

FIGURE 15.—Pigment cells in the skin from the ear of a 25-day-old rabbit; stained with orcein and Giemsa: *pc*, Pigment cells in stratum germinativum; *ctl*, cartilage; *d*, dermis; *ep*, epidermis.   × 100.

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mouse.　Whether or not these pigmented dermal cells are true melano-blasts is uncertain.　Laidlaw (*24*) states that the pigmented cells in the dermis are cells that have taken up, or phagocytized, melanin on their way to the lymphatics.

DOPA, THE TEST FOR MELANIN

Melanin, because it is highly insoluble, is difficult to isolate.　Many substances have been proposed as possible substrates, among which are derivatives of pyrocatechin (*6*) and pyrrol.　The most widely accepted hypothesis today is that the formation of melanin is an oxidative process in which an enzyme in the pigment cell itself acts on a substance brought to the cell via the circulating blood.　Bloch (*5*) and his supporters (*2, 26*) call the enzyme dopa-oxidase and consider the melanogen, or colorless precursor, to be a substance closely related to dopa.　However, the specificity of the enzyme has been questioned (*4*) since the reaction is also given by some tissue elements not con-nected with pigment formation, such as parts of the sweat gland and leucocyte granules.

The purpose of the dopa reaction is to make evident the production capacity of melanin in the skin or hair.　At the time this investigation was undertaken, it was intended to apply the dopa solution to tissue sections of several fetal stages to observe, if possible, the time of pigment determination in the rabbit.　However, the material worked with first was of the adult type, and when this work was completed the supply of dopa powder on hand was exhausted.　A new supply of the powder was unobtainable.　Reed and Sander (*30*) found from grafts
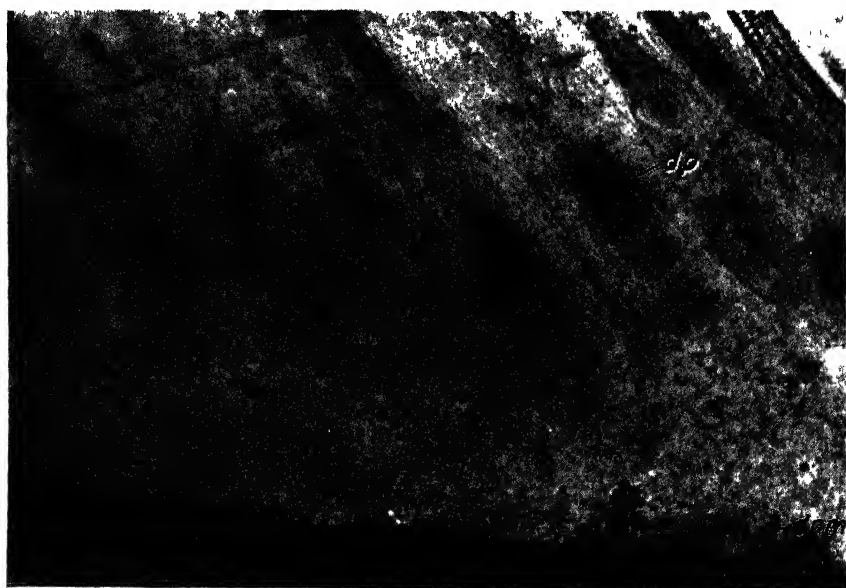
Figure 16.—Longitudinal section of 4-day-old rabbit skin from the loin, treated with dopa: *dp*, Dopa-positive hair bulbs; *dpm*, dopa melanin in dermis.　× 100.

FIGURE 17.—Longitudinal section of skin from adult rabbit shoulder, cut on the freezing microtome and treated for the dopa reaction: *ep*, Epidermis; *du*, upper level of dermis; *dl*, lower level of dermis.   × 100.

that, although pigment granules in the mouse appear on the twenty-first or twenty-second day, the pigmentation pattern is determined by the seventeenth or eighteenth day.

The dopa solution when applied to adult skin tissue, in the manner previously described, causes a good deal of nonspecific blackening, especially around the outer edges of the tissue.   The test is positive in the bulbs of growing hairs (fig. 16) and negative in mature or shedding hairs.   The amount of melanin produced by the dopa reaction is not necessarily the amount normally activated.   The longer the tissue is immersed in the solution, the darker will be the resulting reaction.

Dopa melanin was also observed in the lowermost layer of the epidermis and in the upper and lower levels of the dermis (fig. 17).   The significance to be attached to the reaction in the dermis is difficult to interpret since no dermal pigment was observed in any of the slides made of nontreated tissue.   In some cases, when blood vessels or capillaries were present, it was noticed that the dermal dopa pigment tended to concentrate near these structures.

SUMMARY

In a study of the sequence of pigment development in the short-haired rabbit of the agouti or barred pattern, skin sections were made of two fetuses 18 and 25 days old, nine juveniles from 1 to 25 days old, and one adult rabbit.

No histological evidence of pigment was found in the 18-day-old fetus; hair primordia, however, were clearly established.   The first indications of the pigment cells, the melanoblasts, show up in sections

of the hair of the 25-day-old fetus. These cells are rounded in form and are located in the matrix of the hair bulbs.

Rabbits are covered with a good growth of hair within approximately 5 days after birth. The development of melanin proceeds rapidly and the pigment cells, as they are moved upward from the bulb, take part in the formation of the medulla and the cortex. Only granular pigment was observed in the medulla, whereas both granular and diffuse pigments are present in the cortex. By the time the hairs approach the level of the skin, the nuclei have become smaller owing to a shrinkage of the cytoplasm and air spaces appear between the cells of the medullae. This phenomenon is responsible for the ladder-like appearance of the medullary cells.

The orientation of pigment granules to form a cap above, and perhaps in some cases below, the nucleus in the medullae of rabbit hairs may be influenced to some extent by the cellular polarity of the melanoblasts.

The difference in intensity of melanin at the time the granules are laid down accounts for the differences in color of the fibers.

In the unprime pelt, pigment spots are indicative of the deposition of melanin in new hairs that are being formed just below the skin surface. Mature hairs contain no pigment at the root end, and it is for this reason that the skin of a prime pelt is creamy white and free from pigment spots.

When the dopa test, devised by Bloch to check on the potentialities of pigment development, was used, the reaction in the adult rabbit was positive in the bulbs of the growing hairs and negative in mature, or shedding, hairs. Dopa melanin was present in the dermis and in the lowermost layer of the epidermis of the dopa-treated sections. However, in the untreated sections no melanoblasts were found in the skin of the rabbit except in the basal layer of the epidermis of the ear.

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COMPLEMENTARY FACTORS FOR DARK-RED PLANT COLOR IN UPLAND COTTON [1]

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INTRODUCTION

Red plant color in upland cotton (*Gossypium hirsutum* L.) was found by McLendon (*3*)[3] and Ware (*8*) to be due to a single incompletely dominant factor. A series of allelomorphic factors governing intensity and distribution of red color, or anthocyanin pigmentation, in *G. hirsutum* and other New World ($n=26$) cottons was later reported by Harland (*1*), who found that the factor for *hirsutum* red was not, however, a member of this series but was a duplicate gene. In the Asiatic ($n=13$) cottons the gene for red plant color was reported by Silow and Yu (*6*) to be a member of an extensive anthocyanin allele series.

A red plant-color mutation originating as a red chimera on a normal green plant of the Acala variety of upland cotton at Shafter, Calif., was described by McMichael (*4*). He found that dwarfing was apparently completely associated with the red plant color and that the F_2 from a cross of the dwarf red mutant with normal green plants segregated into a 1 : 2 : 1 ratio, demonstrating incomplete dominance for dwarfing and red color. Another dwarf red mutation occurring as a red chimera on a normal green plant of the upland variety Cook 912 was found by D. M. Simpson at Knoxville, Tenn., in 1939. The former mutation is designated as dwarf red Acala and the latter as dwarf red Cook 912.

The present paper describes a dark-red plant color which appeared in a stock of red Acala cotton of normal stature at Stoneville, Miss., and presents the results of studies which show that the inheritance of dark red may be interpreted in terms of the factor for *hirsutum* red and a factor for dwarf red.

MATERIALS AND METHODS

A stock of red Acala cotton of normal stature was transferred from Shafter, Calif., to Stoneville, Miss., and was planted there in 1937. The following year a progeny obtained by selfing a single plant of this stock contained two darker red segregates, which on continued selfing and selection produced a homozygous phenotype referred to here as dark red. The homozygous plants were about half the usual height of green or red Acala. They were of a greatly intensified blackish-red anthocyanin color throughout (including the bolls). The dorsal surface of the leaves was deep red in contrast to the green dorsal surface in red Acala and other red strains of cotton.

Dwarf red Cook 912 was introduced to Stoneville in 1939, and dwarf red Acala in 1942. These stocks were similar to dark red in plant size but were lighter pigmented, being of a color lighter than *hirsutum* red as represented by the red Acala and Winesap stocks. Dwarf red Cook 912 was somewhat smaller and darker red than dwarf red Acala.

In 1944 a single plant of dark red used as the pollen parent was crossed with normal green Deltatype Webber, an upland variety. The same dark-red plant was crossed with the linkage tester $R_1^{RO}L^o$ $Lc_1^K Fn$. (In conformity with the symbols proposed by Hutchinson and Silow (2), R_1^{RO} here designates red plant color, L^o okra leaf shape, Lc_1^K brown (khaki) lint color, and Fn naked (not fuzzy) seed.) The F_1 progenies from these crosses were selfed and were backcrossed, in the first case to normal green and in the second case to the linkage tester. The F_2 and backcross populations were classified as to plant color before the stands were thinned and again after the thinning. Later the F_2 and backcross progenies from the crosses involving dark red and the linkage tester were classified as to all the characters of the tester.

Near the end of the growing season, plant height measurements were made in the F_2 population from the cross dark red $\times$ normal green Deltatype Webber to evaluate the dwarfness which appeared in some of the color phenotypes.

To check the F_2 classification in both series of crosses, all plants were selfed. For each phenotype the number of progenies grown in the F_3 was as far as possible proportionate to the observed frequency in the thinned F_2 stand, the desired number of progenies for the F_2 class lowest in frequency being 10. The F_3 progenies were classified as to plant color only.

In 1945 dwarf red Acala and dwarf red Cook 912 were crossed with the linkage tester. The F_2 population from each of these two crosses was classified as to plant color. The populations obtained by backcrossing the F_1 of each cross to the tester were classified as to both plant color and leaf shape.

In the same year dwarf red Acala was crossed with dwarf red Cook 912. The F_1 population from this cross was compared with the parental stocks as to plant height and plant color. The F_2 population was classified as to plant color only.

INHERITANCE OF THE DARK-RED CHARACTER

F₂ AND BACKCROSS TESTS

Dark red when crossed with normal green Deltatype Webber produced an F_1 about intermediate in height between the two parental stocks but having a red color about intermediate between that of red Acala and dark red. In the F_2 seven color phenotypes were distinguished. These and their plant frequencies are given in table 1.

The plants of the red phenotype resembled red Acala. The dwarf red plants, the red factor of which is designated by the symbol Rd, were somewhat lighter than the red in color and differed from them in pigment distribution, having less color on the leaf blades, especially around the stomata, but having deeper red petioles and leaf veins. The green plants resembled the normal green parent. With respect to color intensity the intermediate-red phenotype fell between the red and the green and the intermediate-dwarf-red phenotype fell between the dwarf-red and the green. The intermediate-dark-red phenotype represented the recovered F_1 type and was distinctly darker than either the dwarf-red or the red. The dark-red phenotype showed somewhat more variation in color and in plant height than the parental type.

TABLE 1.—*Observed plant frequencies, mean plant heights near end of growing season, theoretical genotypes and genotype ratio, and Rd factor classes [1] for 7 F₂ phenotypes from the cross dark red × normal green*

F₂ phenotype	Observed plant frequency	Mean plant height	Theoretical genotype	Genotype ratio	Rd factor class
	Number	*Inches*			
Dark red	146	31.53±0.69	$\begin{cases} R_1{}^{RO}R_1{}^{RO}Rd\,Rd \\ R_1{}^{RO}R_1{}^{RO}Rd\,rd \\ R_1{}^{RO}r_1{}^{RO}Rd\,Rd \end{cases}$	$\left.\begin{matrix}1\\2\\2\end{matrix}\right\}5$	[2] 2
Intermediate dark red	196	40.46± .48	$R_1{}^{RO}r_1{}^{RO}Rd\,rd$	4	1
Red	60	52.80± .98	$R_1{}^{RO}R_1{}^{RO}rd\,rd$	1	0
Intermediate red	102	54.32± .77	$R_1{}^{RO}r_1{}^{RO}rd\,rd$	2	0
Dwarf red	26	28.12±1.90	$r_1{}^{RO}r_1{}^{RO}Rd\,Rd$	1	2
Intermediate dwarf red	85	40.40± .83	$r_1{}^{RO}r_1{}^{RO}Rd\,rd$	2	1
Green	69	52.42±1.03	$r_1{}^{RO}r_1{}^{RO}rd\,rd$	1	0

[1] *Rd* factor class = average number of *Rd* (dwarf-red) factors.
[2] Theoretical average number of *Rd* factors is 1.6. It was rounded for convenience.

The theoretical F_2 genotype ratio presented in table 1 was calculated on the assumption that 2 pairs of complementary factors for anthocyanin color were involved, 1 pair in the homozygous condition $(R_1{}^{RO}R_1{}^{RO})$ giving red, the other pair in the homozygous condition $(Rd\,Rd)$ giving dwarf red, and 1 member of each in the double-heterozygous condition $(R_1{}^{RO}r_1{}^{RO}Rd\,rd)$ giving the more intense color intermediate dark red, and the further assumption that plants with 3 color factors $(R_1{}^{RO}R_1{}^{RO}Rd\,rd$ or $R_1{}^{RO}r_1{}^{RO}Rd\,Rd)$ were not distinguished from those with 4 color factors $(R_1{}^{RO}R_1{}^{RO}Rd\,Rd)$. While the observed frequencies approach this ratio, the obtained χ^2 value, 57.96, shows a poor fit $(P=$less than 0.01). However, from a backcross of the F_1 $(R_1{}^{RO}r_1{}^{RO}Rd\,rd)$ to the normal green parent $(r_1{}^{RO}r_1{}^{RO}rd\,rd)$ frequencies of 27 intermediate dark red, 33 intermediate red, 35 intermediate dwarf red, and 29 green were obtained. These frequencies show a good fit $(\chi^2=1.29, P=0.50–0.95)$ to the 1 : 1 : 1 : 1 ratio expected from the segregation of 2 independent pairs of factors.

It may readily be determined from table 1 that the dwarf-red and dark-red phenotypes alone fell short of the expected frequencies. This, undoubtedly, was due largely to the failure of some plants with two *Rd* factors to survive to the time of classification. This fact alone could account for the poor fit of the F_2 ratio. The backcross progeny, on the other hand, did not include any phenotypes homozygous for *Rd*. To obviate similar difficulties in the next generation, F_3 progenies were classified as early as practicable.

Measurements taken near the end of the growing season showed the phenotypes to differ in plant height (table 1). The analysis of variance (table 2) brings out that these differences were highly significant ($F=142.73$). From table 1 it appears that the differences in plant height between phenotypes were associated with differences in number of *Rd* factors involved, the phenotypes with no *Rd* factors (class 0) having the greatest plant height while those with two *Rd* factors (class 2) had the least. The analysis of variance (table 2) shows, in fact, that the differences in plant height between phenotypes were due almost entirely to height differences between *Rd* factor classes, which were highly significant ($F=424.58$) while height differences between phenotypes within *Rd* factor classes were not significant ($F=1.80$). This suggests that dwarf stature is closely associated with this factor for red plant color.

TABLE 2.—*Analysis of variance for plant heights of the F_2 population from the cross dark red × normal green*

Source of variation	Degrees of freedom	Variance	F value
Between phenotypes	6	8,589.22	142.73**
Between *Rd* factor classes	2	25,551.32	424.58**
Within *Rd* factor classes	4	108.17	1.80
Within phenotypes	677	60.18	
Total	683		

**Significant at the 1-percent point.

The dark-red phenotype, classed as having 2 *Rd* factors (table 1), theoretically averaged only 1.6, because two plants out of five were of the genotype $R_1^{RO}R_1^{RO}Rd$ *rd*. The difference of 0.4 factor between 1.6 and 2, the number of *Rd* factors for the dwarf-red phenotype, was associated with a mean difference in plant height of 3.41 inches, which in terms of its standard error is not significant ($t=1.69$).

The F_2 from the cross of dark red with the linkage tester consisted of 205 dark-red and 80 red plants, frequencies showing a good fit ($\chi^2=1.43$, $P=0.20-0.50$) to the 3:1 ratio expected where dark red and the linkage tester are both homozygous for red. These results identified the red factor involved in dark red with that for *hirsutum* red, R_1^{RO}, previously reported by McLendon (*3*), Ware (*8*), and others under different symbols.

F_3 PROGENY TESTS

For 4 of the F_3 progenies from the cross of dark red with normal green, it was observed that the F_2 phenotype classification had been incorrect. Out of 9 progenies from plants classed as dwarf red, 3

segregated as intermediate dwarf red; and out of 36 progenies from plants classed as intermediate dwarf red, 1 proved to be of dwarf red parentage. The failure to distinguish accurately between these phenotypes probably explains the high standard error of the mean height shown for dwarf red in table 1. The results of classifying the F_3 progenies other than the 4 misidentified are presented in table 3.

As expected from the genotypes postulated in table 1, three of the phenotypes listed in table 3—red, dwarf red, and green—showed no segregation in the F_3. The intermediate red and intermediate dwarf red each segregated into three phenotypes, the frequencies of which showed a close fit to the ratio 1:2:1. The intermediate dark red segregated into seven phenotypes, the frequencies of which showed a good fit to the ratio 5:4:1:2:1:2:1. The good fit in this case, contrasting with the poor fit of the F_2 frequencies to the same ratio, is attributed in part to the fact that the F_3 progenies were classified before the effect of the differential in survival among genotypes became pronounced.

TABLE 3.—*Observed distribution of plants of F_3 progenies from 7 F_2 phenotypes from the cross dark red × normal green and χ^2 tests for goodness of fit to ratios expected on the basis of duplicate factors for red*

F_2 phenotype	F_3 prog- enies	F_3 plants of indicated phenotype							Theoretical ratio	χ^2	P
		Dark red	Inter- medi- ate dark red	Red	Inter- medi- ate red	Dwarf red	Inter- medi- ate dwarf red	Green			
	Num- ber	*Num- ber*	*Num- ber*	*Num- ber*	*Num- ber*	*Num- ber*	*Num- ber*	*Num- ber*			
Dark red........ {	1	7	0	0	0	0	0	0			
	13	183	0	57	0	0	0	0	3:1	0.20	0.50-0.95
	5	40	0	0	0	18	0	0	3:1	1.13	.20- .50
Intermediate dark red	52	460	335	105	186	75	161	79	5:4:1:2:1:2:1	9.72	.10- .20
Red	23	0	0	722	0	0	0	0			
Intermediate red....	38	0	0	278	635	0	0	302	1:2:1	3.44	.10- .20
Dwarf red.. ...	1 6	0	0	0	0	117	0	0			
Intermediate dwarf red	2 35	0	0	0	0	254	590	280	1:2:1	3.99	.10- .20
Green..	25	0	0	0	0	0	0	735			

[1] 3 additional progenies from plants classed as dwarf red in the F_2 segregated as intermediate dwarf red.
[2] 1 additional progeny from a plant classed as intermediate dwarf red in the F_2 proved to be dwarf red.

Of 19 F_3 progenies from dark-red F_2 plants, 1 did not segregate. The 7 plants in this progeny were all dark-red dwarfs. Thirteen progenies from dark-red F_2 plants segregated for dark red and red, in frequencies showing a good fit to a 3:1 ratio. On closer inspection these 13 progenies were found to be separable into 3 classes—66 dark-red dwarfs, 117 lighter dark-red intermediate dwarfs, and 57 red nondwarfs—frequencies which show a good fit to the ratio 1:2:1 ($\chi^2=0.82$, $P=0.50-0.95$). The frequencies of the remaining 5 progenies from dark-red F_2 plants showed a good fit to a 3:1 ratio for dark red and dwarf red. On closer study these 5 progenies were found to be separable into 3 classes—12 dark-red dwarfs, 28 lighter dark-red dwarfs, and 18 dwarf reds—frequencies which show a good fit to the ratio 1:2:1 ($\chi^2=1.31$, $P=0.50-0.95$). These results for the 3 types of progenies from dark-red plants would be expected

from the genotypes postulated in table 1 on the assumption that dwarf stature and red plant color in the dwarf-red phenotype were due to a single factor or to completely linked factors.

According to the theoretical ratios given in table 1 the expected distribution of a total of 19 F_3 progenies among the 3 genotypes $R_1^{RO}R_1^{RO}Rd\ Rd$, $R_1^{RO}R_1^{RO}Rd\ rd$, and $R_1^{RO}r_1^{RO}Rd\ Rd$ would approximate the ratio 4:8:8 if the 3 genotypes were equal in capacity to survive and mature seed. The observed frequencies, 1:13:5, agree with the results for the dwarf-red phenotype in showing lower capacity to reproduce on the part of the genotypes which were homozygous for Rd.

Results of the F_3 tests support the tentative finding based on F_2 and backcross tests that the dark-red character is due to the complementary action of two factors affecting anthocyanin pigmentation, one of these, R_1^{RO}, being a factor for red and the other, Rd, being a factor for dwarf red.

TESTS FOR INDEPENDENCE OF THE DWARF-RED FACTOR

Absence of linkage between R_1^{RO} and Rd is evidenced by the F_3 segregation of the intermediate-dark-red phenotype (table 3), the χ^2 value for which shows a good fit to the ratio expected on the basis of independent inheritance.

Additional tests for independent inheritance of dwarf red were available from the cross of dark red with the linkage tester. Incomplete dominance of L^O and Lc_1^K over their normal allelomorphs and the independent inheritance of these factors, demonstrated by Ware (9, 10), were confirmed by significant χ^2 values for goodness of fit to the monohybrid and dihybrid ratios involved. Dominance of Fn, reported by Thadani (7) and later workers, and this factor's independence of the three others were similarly confirmed. For convenience in applying the χ^2 tests to determine independence of dwarf red, the factors L^O and Lc_1^K were treated as dominants by including intermediate-okra-leaf plants in the okra classification and including intermediate-brown-lint plants in the brown classification (table 4).

TABLE 4.—χ^2 *tests for the dwarf-red factor's independence of 3 factors of the linkage tester*

[Calculated frequencies based on the observed single-factor segregation in the F_2 from the F_1 hybrid $R_1^{RO}R_1^{RO}\ Rd\ rd\ L^O\ l^O\ Lc_1^K\ lc_1^K\ Fn\ fn$]

	Plants of indicated phenotype				χ^2	P
Segregating factors	Observed		Calculated			
	Dark red	Red	Dark red	Red		
	Number	*Number*	*Number*	*Number*		
Rd and L^O:						
Okra [1]	49	40	48.76	40.24	} 0.01	0.50–0.95
Normal	14	12	14.24	11.76		
Rd and Lc_1^K:						
Brown [2]	46	39	46.57	38.43	} .06	.50– .95
Normal	17	13	16.43	13.57		
Rd and Fn:						
Naked	41	41	44.92	37.08	} 2.64	.10– .20
Normal	22	11	18.08	14.92		

[1] Includes intermediate okra.   [2] Includes intermediate brown.

Thinning the F_2 population to a single plant per hill altered the ratio of dark-red and red plants (originally 3:1). This invalidated the theoretical 9:3:3:1 dihybrid ratio for testing the dwarf-red factor's independence of the factors of the linkage tester. The observed frequencies were, therefore, tested against frequencies calculated from the observed single-factor segregation of the two pairs of genes in the manner described by Mather (5).

Since all F_2 plants were homozygous for $R_1{}^{RO}$, the presence of Rd, either homozygous or heterozygous, resulted in a dark-red phenotype and its absence resulted in a red phenotype. In table 4, therefore, the dwarf-red factor's independence of the factors of the linkage tester is phenotypically expressed as the independent segregation of dark red. The observed phenotype frequencies do not differ significantly from those expected on the assumption that Rd is inherited independently of L^O, $Lc_1{}^K$, and Fn.

DWARF-RED CHARACTER IN ACALA AND COOK 912

Dwarf red Acala and dwarf red Cook 912 crossed with the linkage tester $R_1{}^{RO}L^OLc_1{}^KFn$ gave F_1 progenies which in both cases were darker red than either parent. The F_2 from each cross segregated into seven plant-color classes similar to those of the F_2 from dark red $\times$ normal green. Tests for goodness of fit to the 5:4:1:2:1:2:1 ratio expected on the basis of independent inheritance resulted as follows: for the dwarf red Acala cross, $\chi^2=3.22$, $P=0.50-0.95$; for the dwarf red Cook 912 cross, $\chi^2=15.36$, $P=0.01-0.02$.

The backcross to dwarf red Acala of the F_1 from dwarf red Acala $\times$ $R_1{}^{RO}L^OLc_1{}^KFn$, on the assumption of independent inheritance, would be expected to give dark red, intermediate dark red, dwarf red, and intermediate dwarf red in the ratio 1:1:1:1. The frequencies of a 310-plant population produced by such a backcross showed a good fit to the expected ratio ($\chi^2=5.0$, $P=0.10-0.20$). The frequencies of a 454-plant population produced by a parallel backcross involving dwarf red Cook 912 likewise showed a good fit to the 1:1:1:1 ratio ($\chi^2=1.14$, $P=0.50-0.95$). These results suggest that the mode of inheritance was the same for both dwarf-red phenotypes.

The two backcross populations were classified also as to intermediate-okra versus normal leaf shape. The observed phenotype frequencies, presented in table 5, show a good fit in each case to the 1:1:1:1:1:1:1:1 ratio expected on the basis of independence of the factor for dwarf red and that for okra leaf. These results agree with those for the Rd factor of the original dark red.

Dwarf red Acala was distinguishable from dwarf red Cook 912, the latter being slightly darker pigmented and smaller. Mean plant heights of unreplicated plantings near the end of the 1946 growing season were 19.4 inches for dwarf red Cook 912, 28.6 inches for dwarf red Acala, and 22.6 inches for their F_1 hybrid. The F_1 showed no complementary effect of color factors. The F_2 phenotype classes were not clearly defined, owing to the close similarity of the parents. Only 3 classes based on color intensity were distinguished, the Acala parent type, an intermediate, and the Cook 912 parent type. Frequencies of 26, 66, and 40, respectively, showed a good fit ($\chi^2=2.97$, $P=0.20-0.50$) to the ratio 1 : 2 : 1, indicating that the 2 dwarf-red factors were allelomorphic.

TABLE 5.—*Observed plant frequencies for 8 phenotypes from the backcross $r_1{}^{RO}$ Rd $l^o/R_1{}^{RO}rdL^o \times r_1{}^{RO}Rd$ $l^o/r_1{}^{RO}$ Rd l^o and χ^2 values for goodness of fit of the joint segregation to the expected 1:1:1:1:1:1:1:1 ratio*

Plant-color and leaf-shape phenotype	Observed frequency of plants from cross involving—	
	Dwarf red Acala	Dwarf red Cook 912
	Number	*Number*
Dark red, normal	45	57
Intermediate dark red, normal	38	57
Dwarf red, normal	43	68
Intermediate dwarf red, normal	48	65
Dark red, intermediate okra	25	58
Intermediate dark red, intermediate okra	30	47
Dwarf red, intermediate okra	36	51
Intermediate dwarf red, intermediate okra	45	51
Total	310	454
χ^2	11.75	6.30
P	0.10-0.20	0.50-0.95

Dwarf red Acala was indistinguishable from the dwarf-red phenotype which segregated out of the original dark red. This fact, together with the evidence from the F_2 and backcross tests, suggests that in these two cases the dwarf-red factors may be identical.

SUMMARY

A dark-red, dwarf phenotype here designated as dark red was isolated at Stoneville, Miss., from a culture of red Acala cotton.

Crossing dark-red with normal green cotton gave seven readily distinguishable color classes in the F_2, whose frequencies suggested that dark red was due to the complementary action of two pairs of factors $(R_1{}^{RO}R_1{}^{RO}RdRd)$. Observed plant frequencies in a four-color classification of the progeny obtained by backcrossing the F_1 to normal green showed a good fit to the 1:1:1:1 ratio expected from the segregation of two independent pairs of factors.

In the F_2, significant differences in plant height appeared between phenotypes which differed in number of Rd (dwarf-red) factors, but not between phenotypes which had the same number of Rd factors. The contrast suggests that dwarf stature is closely associated with this factor for red plant color.

F_2 classifications and postulated genotypes were verified by F_3 data, which showed good fit to expected ratios for three F_2 phenotypes and no segregation for three others presumed to be homozygous. One phenotype was shown to consist of three genotypes as postulated.

Results of crossing dark red with the linkage tester $R_1{}^{RO}L^oLc_1{}^KFn$ identified the red-plant factor involved in dark red with the factor $R_1{}^{RO}$ and showed the dwarf-red factor to be independent of the factors for okra leaf, brown lint, and naked seed.

When dwarf red Acala and dwarf red Cook 912, both of which originated as red chimeras on normal green plants, were crossed with the linkage tester bearing the factor $R_1{}^{RO}$, they segregated in the F_2 into seven color classes similar to those obtained by crossing dark red with normal green. Results from the backcrosses of the F_1 prog-

enies to the dwarf red parents showed the dwarf-red factor in each case to be independent of the factor for okra leaf.

The frequencies of the F_2 phenotypes from dwarf red Acala $\times$ dwarf red Cook 912 showed a good fit to the ratio 1 : 2 : 1, indicating that the dwarf-red factors in these two stocks were allelomorphic.

It is suggested that dwarf red Acala and the dwarf-red phenotype which segregated out of dark red may be controlled by identical factors.

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